Acta Oncologica Vol. 36, No. 6, pp. 658–665

Abstracts of Theses from the Scandinavian Countries

Abstracts of Scandinavian theses on oncologic subjects are published under this heading. The full theses are as a rule published by the universities or as supplements to different journals. They can usually be obtained after contact with the author.

Studies on Krüppel-related zinc finger proteins and their involvement in normal and malignant hematopoietic growth and differentiation

MAGNUS ÅBRINK

Department of Medical Immunology and Microbiology, Uppsala University, BMC, Box 582, S-751 23 Uppsala, Sweden

The family of Krüppel-related zinc finger proteins, constitute with its 500–1000 members probably the largest family of nucleic acid binding proteins in the human genome. Several members of this gene family have been shown to be important regulators of hematopoietic growth and differentiation. However, the majority of the zinc finger proteins have not been characterised structurally, nor have their biological functions been determined. To identify novel Krüppel-related zinc finger proteins, involved in human monocyte-macrophage differentiation, a cDNA library from the human monoblast cell line U-937 was screened with a degenerate oligonucleotide probe directed against the zinc finger motif. Sixty-three independent cDNA clones were isolated. Nucleotide sequence analysis showed that these clones originate from approximately 42 different zinc finger genes, representing 35 novel genes as well as 7 previously characterised zinc finger genes. Northern blot analysis showed that most of these clones encoded proteins that showed ubiquitous tissue distribution. However, HZF 5, 12, and 13 exhibited interesting expression patterns. HZF 5 was down-regulated during terminal differentiation. The expression of HZF 12 was restricted to the testis. HZF 13 was identical to a previously described zinc finger gene, H-plk. Due to an insertion of an endogenous retrovirus (ERV3) in the 5′ end of the zinc finger gene, this gene is regulated by the 5′LTR of this retrovirus. The expression of H-plk is high in steroid dependent organs and in the monoblast cell lines U-937 and THP-1. We showed that the restricted expression in monocytic cell lines of HZF 13 is dependent on specific demethylations downstream of the 5′LTR of the retrovirus. By genomic Southern blotting, PCR amplification and sequence analysis of the obtained products we were able to show that this demethylation was restricted to cells of the monocyte cell lineage. The demethylation affected at least three independent CpG dinucleotides in this region of the gene, and the methylation status was also shown to correlate with the transcriptional activity of the gene.

To establish an in vitro model for studying the expression of zinc finger genes during monocyte development, three independent monocytic cell lines were studied for their phenotype and degree of differentiation. The cell lines, U-937, THP-1 and Mono Mac 6, which previously have been described as being arrested during various stages of monocyte differentiation, were all shown to represent relatively immature cells. This conclusion was based on the expression of several differentiation markers expressed only during the myelocyte-monoblast stage of differentiation, e.g. the serine proteases, cathepsin G, proteinase 3, and N-elastase as well as myeloperoxidase and azurocidin. In addition, the cells lacked expression, or expressed only low amounts of several markers characteristic of more mature cells of this cell lineage, e.g. the differentiation markers, lysozyme, CD14, and CD23. Blood monocytes express all of these markers at high or intermediate levels. The immature phenotype of all three cell lines indicates that the monoblasts/monocytes are particularly sensitive to neoplastic transformation at a stage of high proliferative potential during the early stages of differentiation. In addition, expression of the mast cell tryptase was detected in two out of three monocytic cell lines and its expression was shown to be upregulated by the phorbolester PMA. The tryptase may represent a novel phenotypic marker for the identification of monocytic leukemias.

January 1997

Studies on genes involved in tumorigenesis and metastasis of human melanoma and sarcoma

GUNHILD MARI MÆLANDSMO

Department of Tumor Biology, Institute for Cancer Research, the Norwegian Radium Hospital, and Faculty of Medicine, University of Oslo, Oslo, Norway

The following section called future prospects and conclusions is quoted:

Our studies have demonstrated the absence of INK4A mRNA expression in a substantial number of the examined melanoma biopsies, even though gene abnormalities could not be detected. Methylation of a 5′-CpG island in the promoter region of the gene has been suggested as an alternative mechanism for inactivation of the gene, and studies to investigate this possibility in the melanomas should be initiated. Recently, Glendening et al. (1995) reported homozygous loss of the INK4B gene without affecting the closely located INK4A gene during tumor progression in a patient with sporadic melanoma. This observation makes it of interest to hybridize DNA from our melanoma and sarcoma samples with an INK4B probe, and to compare the deletion frequencies of the two INK4 genes, which encode closely related cell inhibitors. Moreover, a point mutation in CDK4, observed both in sporadic and familial melanoma, has been suggested as an alternative mechanism for inactivation of the 'pRb pathway'. This possibility might be examined using our material in attempts to help elucidate the intricate mechanisms of cell cycle regulation and its involvement in cancer.

To further explore the selective importance of different S100 proteins in the development and progression of malignant melanomas the production of specific monoclonal antibodies is essential. Such antibodies may help determine whether S100L staining is valuable in distinguishing between benign and malignant melanocytic lesions, and elucidate whether cacy may be applied as a progression marker. Some monoclonal antibodies claimed to be specific for particular S100 proteins are commercially available. We have tested one such S100L antibody without obtaining the desired specificity. Therefore, conclusions concerning the antigen expression on melanocytes compared to melanoma cells, and whether the loss of S100L may be useful in monitoring tumorigenesis and as a marker for early detection of melanoma development, must be drawn with caution.

As is the case for the putative $p16^{INK4A}$ suppressor gene, it has been suggested that the expression of S100L is regulated by methylation (Lee et al., 1992). It would therefore be interesting to compare the methylation pattern of S100L in benign nevi and malignant melanomas to examine whether hypermethylation downregulates the gene expression in the malignant lesions. Furthermore, transfection of S100L into non-expressing melanoma cell lines, and subsequent examination of the effect on tumor

growth and tumorigenicity, may give some indication whether this S100 protein belongs on the list of tumor suppressors important for melanoma development.

By specific gene inhibition it is possible to obtain valuable new knowledge about the fundamental role of a particular gene product, exemplified by the capl protein and its involvement in metastasis formation. A particularly interesting and still unanswered question is whether capl can induce metastasis in non-metastazing cells. We have recently made an expression construct with human CAPL and are currently transfecting this into non- and low-metastazing cell lines. In such studies it is probably necessary to evaluate the metastasis inducing properties by in vitro invasion-and motility-assays as it may be difficult to measure the metastasis formation capacity of originally non-metastazing cell lines in in vivo model systems. Thus, even if capl has a rate-limiting function in the metastatic process, it is not obvious that such transfected cells will give metastatic disease when tested in vivo. The CAPL specific ribozyme has also been subcloned into an inducible expression vector. We want to cotransfect these two expression constructs and assess the effects of variable CAPL expression levels by addition and removal of the inducer to the double-transfectants. In addition, an adenovirus construct harboring the CAPL specific ribozyme has been made. This construct will be tested in in vitro cell lines, pending sufficient activity it will be assessed in the OHS animal model and examined for possible further application in gene therapy experiments.

In parallel with the ribozyme studies, phosphorothioate modified antisense oligonucleotides against CAPL have been synthesized. Upon delivery of an antisense oligonucleotide directed against the ribozyme recognition sequence preliminary results show a 70–80% reduction of the protein level in one cell line. Experiments evaluating the metastatic capacity upon LV injection of in vitro antisense treated cells have been initiated.

For a variety of different types of cancer good regimens for treating metastatic disease are still lacking. Metastasis is the principle cause of death of cancer patients, and early detection of metastatic tumor cells, development of new treatment strategies, and knowledge about how to prevent cancer metastasis are some of the most challenging tasks in cancer research. On this background, progress in developing new strategies to treat or inhibit metastatic formation are of great clinical interest. Cancer, originating from an accumulation of genetic disorders, is an obvious target for gene modulating experiments. Thus, somatic gene therapy represents a fascinating new approach in cancer research. Antisense oligonucleotides or ribozymes directed against key gene transcripts may prove valuable in future cancer therapy.

The high degree of specificity in causing cleavage of the target transcript, and the potential for limited toxic side effects, have made ribozymes attractive candidates for therapeutic agents. The successful application of the anti-CAPL hammerhead ribozyme in our model systems makes the extension of the work towards in vivo therapy in animal models, and possibly in the clinic, attractive. The practical usefulness of ribozymes for in vivo studies has been extensively discussed (Sullivan, 1994; Rossi, 1995; Thompson et al., 1995), and factors such as delivery, turnover, stability, and intracellular localization are all uncertainties that obviously will influence the efficiency of the gene inhibiting agent. Therefore, there are still a lot to be learned and several conditions need to be better characterized before the real potential of ribozyme strategies in cancer treatment can be determined.

At the end of this work a few general conclusions may be drawn. The value of studying the status of several genes and their products on the same well-characterized, and reasonably large, panels of human tumors has been confirmed. The opportunity to study both human tumor cell lines and clinical specimens provided information not attainable on each material separately. The interrelationship between the regulation of individual genes was elucidated. Patterns that could not have been foreseen became apparent, and a few pieces in the puzzle seemed to fall into place. That two different tumor types were studied revealed distinct effects of the genes in tumors of dissimilar origin. Distinction between groups of genes involved in cancer is important from a mechanistic point of view. However, alterations in genes regulating the cell cycle, cell proliferation, cytoskeletal organization, cell movement, and metastasis formation are all involved in determining the aggressiveness of the tumor and the progression of the disease, although the relative importance differ. Work to further elucidate the complex relationship and interaction between such genes and their products seems to be an intriguing and challenging task in the years to come.

January 1997

Sialyl-Lewis a-carrying mucins secreted from colon carcinoma bind to E-selectin and inhibit leukocyte adhesion to E-selectin expressing cell and natural killer cytotoxic lysis

KE ZHANG

Department of Medical Biochemistry, Göteborg University, Medicinaregatan 9A, S-413 90 Göteborg, Sweden

Aim: The aim of the studies was to investigate the interaction between sialyl-Lewis a-carrying mucins purified from a colon carcinoma cell line and E-selectin and the effect of the interaction on leukocyte adhesion to E-selectin, to study the effect of the sialyl-Lewis a-carrying mucins from colon cancer patient serum on leukocyte adhesion to E-selectin, and to observe the relation between clinical outcome and the profile of sialyl-Lewis a carrying mucins in the patient sera.

Methods: Sialyl-Lewis a-carrying mucins have been purified from large scale carcinoma cell culture in roller bottles using trichloracetic acid precipitation, ultra filtration, affinity chromatography and gel filtration. Sialyl-Lewis a-carrying mucins from colon cancer patient sera were purified in a similar way. These purified mucins or colon cancer sera were tested for binding to E-selectin transfected cells or to purified E-selectin-Fc fusion protein, and used in cell adhesion assays to inhibit leukocyte binding to E-selectin-expressing cells including IL-1β induced endothelial cells.

Results: Two secreted mucin-type glycoproteins produced by the colon carcinoma cell line COLO 205, H-CanAg (MUC1 as apoprotein) and L-CanAg (CD43 as core protein) were purified from spent culture media. H-CanAg expressed sialyl-Lewis a and x, while L-CanAg only expressed sialyl-Lewis a. They could bind to E-selectin transfected cells in a Ca^{2+} and E-selectin dependent way. These mucins could inhibit leukocyte adhesion to E-selectin expressing cells and the inhibition could be blocked by antibodies against sialyl-Lewis a. H-CanAg could bind to E-selectin and inhibit leukocyte adhesion more efficiently than L-CanAg.

Colon cancer sera with high levels of sialyl-Lewis a and purified sialyl-Lewis a carrying mucins from these sera of patients with advanced disease could inhibit leukocyte adhesion to E-selectin, proposing that the levels of mucins in their circulation could be sufficient to impede leukocyte adhesion. The sialyl-Lewis a-carrying mucins in colon cancer patient serum had different molecular sizes as shown by gel filtration. The larger-sized mucins, containing MUC1, expressed both sialyl-Lewis a and x, and the small-sized mucins expressed sialyl-Lewis a only. The cell-adhesion inhibitory effect of the former was stronger than the latter.

The secreted MUC1 mucin purified from COLO 205 cell spent culture media could inhibit target lysis by NK cells. When the target K562 cells expressed higher concentrations of the MUC1 mucin by transfection, these were more resistant to NK lysis compared with control and wild type K562 cells.

By analysing sialyl-Lewis a-carrying mucins purified from colon cancer sera using affinity chromatography and gel fitration, we observed a relation between clinical outcome and the profile of sialyl-Lewis a-carrying mucins, indicating that the profile has a predictive value for the outcome of colorectal cancers.

Conclusions: Sialyl-Lewis a-carrying mucins secreted by a cancer cell line or from colon cancer sera could interact with E-selectin and inhibit leukocyte adhesion. This supports the hypothesis that high local concentration of secreted sialyl-Lewis a-carrying mucins produced by tumour cells could inhibit leukocyte extravasation to the tumour and impede NK-cell mediated lysis. These effcts of secreted mucins could be a way for tumour cells to escape the immune system.

February 1997

The significance and urokinase-type plasminogen activator (u-PA) in tumour growth and Linomide-induced upregulation of u-PA's endogenous inhibitor PAI-2

ANITA BILLSTRÖM

Department of Obstetrics and Gynaecology, University Hospital, S-221 85 Lund, Sweden

The progressive process of tumour invasion and generation of metastases is the primary cause of death for most patients with cancer. Some of the regulatory components of this progressive process are adhesion, migration, and proteolysis. Urokinase-type plasminogen activator (u-PA) is a serine protease associated with tissue remodelling, cellular invasiveness, matrix degradation, angiogenesis, and cell migration. It was therefore deemed important, from both a physiological and a therapeutic point of view, to investigate whether the inhibition of u-PA could suppress tumour growth.

The human prostate cancer cell line DU 145 expresses high levels of u-PA in cell culture, and when the cells were inoculated subcutaneously (sc) into immunodeficient mice they grew easily as xenograft and retained their ability to produce and secrete u-PA with no detectable levels of tissue-type plasminogen activator (t-PA) or of the plasminogen activator inhibitors PAI-1 and PAI-2. It is thus a suitable in vivo model for investigating the role of u-PA in cancer. The synthetic u-PA inhibitor, p-aminobenzamidine, was found to suppress the growth of DU 145 tumours in this in vivo model as did daily sc. injections of recombinant PAI-2 (rPAI-2), the endogeneous inhibitor of u-PA. Therapeutic administration of rPAI-2 over longer periods of time, however, is hardly feasible; one possible approach was therefore the upregulation of the endogenous PAI-2 production. Linomide® (roquinimex), a compound previously shown to have therapeutic effects in tumour models as well as in models of autoimmunity, was found to upregulate PAI-2 in cultured human peripheral blood monocytes, both at the protein and the mRNA levels. In a study with healthy volunteers, it was shown that PAI-2 levels in monocytes were also enhanced in vivo by Linomide.

Thus, in this thesis it is shown that u-PA is a significant factor for tumour growth and that the production of the u-PA's inhibitor PAI-2 in humans can be upregulated by drugs. One strategy for anti-tumour treatment might therefore be to stimulate the endogenous production of PAI-2 in monocytes/macrophages.

February 1997

Assessing the life situation of children and adolescents with cancer and their families

KARIN ENSKÄR

Department of Medicine and Care, Division of Nursing Science, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden

In recent decades, more children have been cured of cancer due to research development and intensification of treatment. Having a life-threatening disease, like cancer, during childhood effects both the child and the family. It is important for the staff working with those families to know what is distressing in order to provide appropriate care. The aim of this thesis was to develop instruments to assess the life situation of children with cancer and their families. First, the health care personnel's (n = 24) perception of problems and symptoms caused by the disease were identified. An inquiry of Delphi-technique type was used. The results gave two Lists of Problems (LoP), one with 84 problems concerning the child, and one with 69 problems concerning the rest of the family. In the next study, using qualitative interviews, children's (n = 5), adolescents' (n = 10) and parents' (n = 16) own experience of problems related to the disease and its perceived effect on their life situation were investigated. The result from those studies formed the basis for the three scales developed in the study, the Life Situation Scales for Children 7–12 years old (LSS-C), for Adolescents 13–19 years old (LSS-A) and for Parents (LSS-P). A new group of children (n = 15), adolescents (n = 21) with cancer and their parents (n = 110) answered one of the three questionnaires. The psychometric tests of the first version of the life situation scales gave some evidence of validity and reliability. However the scales for children and adolescents, in particular, need to be tested on larger samples. The study indicated how to proceed with further development, as the methods used seemed to be adequate. After further development the tools could be used in research and clinical care evaluations.

March 1997

Activated murine natural killer cells—Morphology, differentiation and tumour interactions in vivo and in vitro

MARTIN L. UNGER

Institute of Anatomy and Cell Biology, Medicinaregatan 3, S-413 90 Göteborg, Sweden

Introduction: In adoptive immunotherapy of cancer, in which lymphocytes such as natural killer (NK) cells are activated by interleukin-2 (IL-2) in vitro and subsequently re-infused into the host, it has been shown that effector cells accumulate in tumours where they establish cell-to-cell contact with tumour cells. In clinical studies both partial reduction and total clearance of the tumour has been observed. However, the exact mechanism behind this effect is as yet unclear. This study investigates the basic characteristics of IL-2 activated natural killer (A-NK) cells, their ability to extravasate, migrate, and establish contact with tumour cells in vitro and in vivo.

Specific aims: 1) to thoroughly describe the differentiation and maturation of A-NK cells in vitro; 2) to explore the migration of A-NK cells in vitro and evaluate how different types of extracellular matrix proteins might influence migration: 3) to describe the infiltration of A-NK cells in experimental tumours in vitro; and 4) to examine the ability of A-NK cells to circulate and accumulate in tumours in vivo.

Materials and Methods: Cell culture, light-, fluorescence- and electron microscopy, immunocytochemistry, ^{51}Cr-release- and cell migration assays. Murine A-NK cells from the C57BL/6 strain and B16 melanoma cells were used throughout the studies.

Results: **A.** The proliferation and subcellular differentiation of A-NK cells has been described in detail by means of light and electron microscopy and flow cytometry. It appears that with increasing age, the amount of specific granules and glycogen accumulation in the cytoplasm increases, resulting in a huge volume expansion of the cells. Cytotoxicity of A-NK cells has been evaluated by ^{51}Cr-release in co-cultures with tumour targets. It was found that cytotoxicity decreases with increasing age of the A-NK cells. Interaction of A-NK cells with target cells also undergoes major alterations, i.e. young A-NK cells (3 d.) form conjugates with target cells whereas older cells (6–9 d.) show a protease-like tumour cell release effect.

B. In an in vitro migration model, the role of extracellular matrix constituents on the migratory behaviour of A-NK cells has been studied. The results indicate that A-NK cell migration is substrate-dependent, i.e. laminin and collagen IV favour a directed migration whereas collagen I does not. Moreover, B16 tumour cell aggregates probably release a humoral factor acting as a chemoattractant on A-NK cells.

C. In vitro, A-NK cells were shown to infiltrate B16 microtumours (melanoma aggregates as well as melanoma cells grown on macroporous carriers (Cultispheres)). After 6–8 h of co-incubation, tumour cell aggregates were dissolved in manner resembling treatment with proteases. Thus, while the A-NK cells induced a separation of closely attached tumour cells, no sign of a directed attack against the target cells, e.g. exocytosis of specific granules, could be verified.

D. Lung metastases were found to become infiltrated to a variable extent by adoptively transferred A-NK cells depending on growth pattern and vascularization of tumour nodules. Tumours with little or no infiltration of A-NK cells grew as compact spheroids with a capsule containing only a few microvessels. Conversely, tumours with a more loose growth pattern and a higher degree of vascularization showed a rich infiltration and accumulation of A-NK cells.

E. Even though better vascularized metastases become better infiltrated than poorly vascularized metastases, no evidence has been found that accumulation of A-NK cells in malignant tissue is dependent upon A-NK cell recirculation. Rather, A-NK cell accumulation in tumours appears to rely on local recruitment of migrating A-NK cells first retained in microvessels in the surrounding tissue.

Conclusions: With increasing age, A-NK cells seem to alter their anti-tumour function from specific cytotoxicity to a more protease-like effect on experimental tumours. Rheological characteristics of both the effector cells and the microcirculation favour entrapment of the transferred A-NK cells in the first microvessels encountered. Therefore, recirculation probably occurs to a very low extent. Hence, accumulation of A-NK cells in tumour tissue seems to depend on migration from surrounding normal tissue. The directed migration of A-NK cells in vitro is dependent on a) constituents of extracellular matrix, and b) presence of tumour cell aggregates. A-NK cell infiltration of tumours in vivo also seems to be dependent on a certain growth pattern of the tumour. Based on the present in vitro and in vivo results, the anti-tumor effect of cellular immunotherapy appears to rely on a series of complicated processes involving especially the actual differentiation of the A-NK cells after IL-2 stimulation during culture, their migratory properties, as well as their ability to elicit cell mediated cytotoxicity. The role of the observed tumour disintegration effect of A-NK cells remains obscure but could lead to increased tumour cell dissemination in vivo.

March 1997

A microcell hybrid based 'elimination test' in the search for malignancy related genes

STEPHAN IMREH

Centre of Microbiology and Tumour Biology (MTC), Karolinska Institute, Stockholm, Sweden

This thesis is focused on the elimination test developed by us (Imreh et al., 1994). The elimination test was designed as a functional test for malignancy related gene identification. Its task is to follow the cytogenetic and molecular alterations on single normal chromosomes transferred into tumor cells. We assume that these modifications when occur regularly may be connected to selective growth advantage providing functions. In our first series of experiments we used MCH903.1, MCH906.8 carrying a cytogenetically intact human chr 3 and MCH910.7, MCH939.2 and MCH924.4 carrying human chromsome 3 that contained the deletions, del(3)(pter-p21.3), del(3)(p21.3–p14) and 3del(p24–p14)(q21–q26). We initiated the first quantitative/functional study by FISH chromosome painting on tumors observing that two normal human chr.3/mouse MCHs lost a cytogenetically intact form of the introduced chr.3 during progressive growth in SCID mice. Only fragments translocated into mouse chromosomes (chimeric translocations) were maintained. PCR-analysis using 20 pairs of primers had revealed a common eliminated region including four markers spanning approx. 40 cM on the 3p24–p21.3 region: THRB (3p24.1–p22), AP20R (3p21.33), D3S32 (3p21.3–p21.2) and D3S1029 (3p21.31–p21.2) in 20 SCID-mouse tumours derived from chr3/mouse MCHs. Control microcell hybrids with chr 1 and 8 were not fragmented during SCID tumor growth. Based on these results we proposed the 'elimination test' as a supplementary method for tumor suppressor gene identification. We expected that it may provide a simpler and more easily interpreted test than direct suppression of tumorigenicity that may be hampered by the accidental loss or mutation of an introduced gene. In order to further narrow down the common eliminated region and to characterize the losses, five MCH lines that carried human chr. 3 were included in a follow up study. MCH903.1, MCH906.8 and MCH910.6 carried a cytogenetically intact human chr 3, MCH910.7 and MCH939.2 carried human chr 3 that contained the deletions, del(3) (pter-p21.3) or del(3)(p21.3–p14), respectively. MCH901 carried a cytogenetically intact human chr 1, MCH240.3 carried 1–3 copies of a cytogenetically intact human chr 13, MCH203.4 carried two copies of human chr 13 as Robertsonian fusion chr (translocated into a mouse chromosome), MCH313.4 carried a cytogenetically intact human chr 17 and MCH904.11 carried a cytogenetically intact human chr 8. MCH-derived SCID-mouse tumors, 22 new and 5 previously reported, were analysed by fluorescence in situ hybridisation (FISH: direct painting, DP and reverse chromosome painting, RP), Southern blotting and PCR. 53 chr. 3-specific markers were tested for the presence by PCR. The common eliminated region designated as CER has been narrowed down from an estimated average spacing of 40 cM to ~ 7 cM. CER included the markers AP20R (3p21.3),

D3S966 (3p21.3–21.3), D3S1029 (3p21.3–21.2) WI-7947 (3p22–p21.3), D3S2354 (3p22–p21.3). D3S32 (3p21.3–p21.2) and B362WB9 (3p21.3–p21.2), and was bordered distally by the D3S1260 marker and proximally by the D3S643 marker. The delineation of 7 cM CER was possible due to the identification (both by PCR and RP of an interstitially retained 3p21.3–21.2 fragment designated later as 'rebox-I' and containing 7 markers on the centromeric verge of the CER. A significant solid tumor LOH cluster concorded with the CER. The known HDs in SCLC lines seemed to flank telomerically and centromerically the CER. In 24 serially passaged SCID derived tumors of MCH910.6 and 906.8 (originally containing intact chr.3), the previous results on the elimination of 3p21.3 segments were confirmed. The 'rebox I' was maintained in tumors but on the telomeric side of the CER, another retained box was found: the 'rebox-A'. In 10 MCH 910.6 derived tumors one or two double minute (dmin) like fragments were observed. By PCR analysis using 24 markers we covered the interval between D3S1611 and D3S1235 (3p22–21.2). D3S32 and D3S2354 are regularly eliminated during in vivo tumor growth whereas the other 22 markers D3S1611, ACAA, D3S1260, WI-692, D3S2343, D3S966, D3S1029, D3S643, WI-2420, MST1, GNA12, D3S1235, D3S1298, GLB1, WI-4193, D3S3658, D3S3559, D3S3678, WI-6400, WI-7947, B362WB9 and D3S10865 are regularly retained. We have defined a common eliminated region (designated as CER1) of approximately 1.6 cM, inside the previously identified CER. CER1 extends to D3S1029 on the telomeric and D3S643 on the centromeric side. While pursuing these studies, we have noticed that segments on the long arm of chr. 3 are frequently retained after SCID mouse passage. Using DP, RP and PCR and 4 successive SCID mouse passages of chr. 3 MCHs concordant results were obtained in the majority of tumors. MCH903.1: intact chr. 3; MCH910.8: del(3)(pter-p21.3): MCH939.2: del (3)(p22–p14); MCH939.3:del(3)(pter-cen). The chr. 3 donors were two normal human diploid fibroblast lines, HFDC (for MCH903.1 and 910.7 designated as A1, A2) and HHW1108 (for MCH939.2 and 939.3 designated as B1 and B2). We have confirmed the preferential loss of 3p and showed that after four SCID mouse passages 8 markers (D3S1282, GLUT2, D3S1262, D3S1314, SST, 924ZO69R, 924ZO66R and D3S1265) are obstinately retained in 17 tumors and 9 single cell clones. There is a common retained region (CRR) on 3-25-qter. Several oncogenes including FIM3, EVI1, BCL6, ETS1, ERM are located within CRR. A remarkable concordance was found between the location of CRR and segmental gains over the same region observed by CGH in uterine cervix carcinoma, ovarian cancer and small cell lung carcinoma. Early in our studies on the role of chr.3 chromosome aberrations in cancer initiation, by radioactive in situ hybridization we have localised the D3F15S2 marker to 3p21.2.p21.1, telomeric to the familial RCC t(3;8) translocation breakpoint concluding that the region affected by this translocation is not identical with the region of 3p most frequently deleted in sporadic RCC.

April 1997

Molecular genetic analysis of human breast cancer

CATHERINE M. PHELAN

Institute for Molecular Medicine, Karolinska Institute, Stockholm, Sweden

Breast cancer accounts for approximately 20% of all female malignancies with hereditary breast cancer being implicated in 5–10% of these cases. Two highly penetrant hereditary breast cancer genes are known; BRCA1 (17q) and BRCA2 (13q), which also confer an increased risk of cancer at other sites. The risk of breast and ovarian cancer in BRCA1 mutation carriers appears to be modified by a number of factors, including parity, age of first birth and position of the BRCA1 mutation. We have shown that there is a 2-fold increased risk of ovarian cancer in BRCA1 carriers with rare alleles of the HRAS1 VNTR versus those with common alleles only. We did not observe a difference in breast cancer risk with the presence of rare alleles. This is one of the first examples of a gene modifying the penetrance of an inherited cancer gene.

The proportion of families attributable to BRCA2 is less than previously estimated. In addition to an early age of onset of female breast cancer (less than 35 years), families with BRCA2 mutations are also at an increased risk for male breast, pancreatic, and prostate cancer. Recurrent mutations in conjunction with a common 13q haplotype have been observed in some individuals suggesting that founder effects are present in the inheritance of this disease. LOH on chromosome 13 in sporadic breast and ovarian tumours has implicated BRCA2 as a candidate TSG. However, despite screening 70 breast and 55 ovarian tumours, we identified very few BRCA2 mutations. This lack of significant mutation in BRCA2 (and BRCA1) in sporadic breast and ovarian tumours suggests that either different mechanisms of tumour suppressor gene inactivation play a role or different genes are involved in the sporadic versus familial cases.

The MDGI gene (1p33–p35) has previously been implicated as a breast cancer TSG. However, we have not identified inactivating mutations in the coding region in a panel of sporadic breast tumours. More recently, it was shown that the MDGI gene expression is shut-off by aberrant methylation in breast carcinomas and cell lines. Thus the MDGI gene may not be the target of LOH on chromosome 1p in breast cancer.

In a study of 1280 breast carcinomas, we found multiple regions of LOH on chromosome 17 involved in human breast cancer. The regions were defined by multiple independent deletions and by statistically significant associations between LOH and specific clinopathological features. These include age of onset, family history of breast cancer, tumour histopathology, tumour size, estrogen receptor (ER) status and occurrence of lymph node or distant metastases. Each of the clinical parameters except distant metastases was significantly associated with loss in specific subregions with ER-negative and ductal tumours showing LOH for markers along the majority of the chromosome.

April 1997

Assessment of diagnostic and prognostic factors in pancreatic duct carcinoma

STEFAN LINDER

Department of Surgery, Stockholm Söder Hospital, Karolinska Institute, S-118 83 Stockholm, Sweden

The diagnosis pancreatic carcinoma is suggested by a combination of clinical and radiological signs. The differential diagnosis vs. chronic pancreatitis may, however, be difficult. Morphological verification of malignancy is therefore necessary before initiating non-surgical therapy. The present study is focused on the use of cytological material in the diagnosis of pancreatic carcinoma and some tumour biological properties that may have a prognostic impact in patients with pancreatic carcinoma.

A series of 334 patients with suspected pancreatic carcinoma underwent percutaneous fine-needle aspiration biopsy (FNAB). A

malignant diagnosis was clenched in 270 and cytology was positive in 187. There were no false positive interpretations in 64 patients without a carcinoma. Thus, the sensitivity, specificity and overall accuracy were 69%, 100% and 75%, respectively. There were no complications or cutaneous metastases. FNAB can therefore be considered a safe and reliable procedure for confirming pretreatment diagnoses of pancreatic carcinoma.

Morphometry was studied as an aid to conventional morphology in cytological material from 100 patients with pancreatic carcinoma and 15 with chronic pancreatitis. There were pronounced differences between the two patient groups and interobserver reproducibility was demonstrated. The results therefore indicate that morphometry may be used as an adjunct to conventional cytology in the diagnosis of pancreatic carcinoma.

DNA ploidy was assessed by performing image cytometry (ICM) on cytological samples from 128 patients with pancreatic carcinoma. There were 39 (30%) DNA diploid, 21 (17%) tetraploid and 68 (53%) aneuploid tumours. Among patients who had undergone resection, the DNA pattern was diploid to a greater extent than among those who received non-surgical treatment. Prognostic information was obtained by DNA ploidy and morphometric variables alone, and in combination.

The proliferating antigen Ki-67 and p53 protein expression were analysed by immunohistochemistry (IHC) performed on formalin-fixed paraffin-embedded tumour material. The p53 protein was expressed in 22 tumours (46%) while 26 (54%) were negative. Immunoreactivity of the Ki-67 antigen was evaluated by scoring and interactive image analyses (the proliferating cell index, PCI, and proliferating cell area, PCA, were calculated). All three variables correlated with survival time but only PCA and p53 protein expression had independent prognostic value.

The expression of extracellular matrix (ECM) proteins and adhesion molecules was studied by IHC performed on frozen sections from 10 pancreatic carcinomas. Normal pancreas tissue served as control material. In contrast to the normal pancreas, the basement membrane in carcinomas was discontinuous as demonstrated by type IV collagen and laminin. These two ECM proteins, together wtih tenascin and vitronectin were present in cancer cell areas of the stroma. Corresponding integrin receptors (α_2, α_3, α_v) were expressed on cancer cells, thus providing possibilities for interactions and increased capacity for local invasiveness.

April 1997

Immunosuppression in childhood malignancy—Aspects on humoral immunity and serum cytokine levels

JONAS ABRAHAMSSON

Department of Pediatrics and Clinical Immunology, Göteborg University, Göteborg, Sweden

The primary aims of this study were to evaluate the humoral immunity and to examine the serum levels of pro-inflammatory cytokines in childhood malignancy. Furthermore, the possibility of utilizing serum levels of interleukin-6 (IL-6) as an early indicator of bacterial infection in neutropenic children was investigated.

Serum immunoglobulin (Ig) levels and lymphocyte reactivity were examined in a cohort of 220 children from diagnosis of malignant disease, up until four years after cessation of therapy. Children with leukemia and Hodgkin's disease (HD) had reduced levels of all Ig isotypes during induction therapy and in all tumor groups a profound depression of IgM was observed throughout treatment extending long after completion of therapy. Lymphocyte mitogenic response was low only in children with actue lymphoblastic leukemia (ALL) at time of diagnosis, but was markedly depressed in children with lymphoma and brain tumors during treatment. At time of diagnosis, children with leukemia had low levels of IgA and IgM, but ELISPOT analysis of bone marrow immunoglobulin secreting cells (ISCs) in 32 children showed that the number of ISCs were not reduced as compared to children with solid tumors ($n = 17$). Chemotherapy rapidly reduced the number of bone marrow ISCs of all isotypes.

Specific levels and functional affinity of antibodies in *E. coli* O and poliovirus type 1 antigens were investigated in 45 children. Both the level and avidity of antibodies to these antigens were increased in children with malignant disease. For poliovirus antibodies this was independent of antigenic exposure implicating that an immune dysregulation affects antibody production in these patients.

Using sensitive immunoradiometric methods, serum levels of tumor necrosis factor-α (TNF-α), IL-6 and interferon-γ (IFN-γ) were determined at time of presentation ($n = 59$) and at onset of fever in children with malignant disease ($n = 110$). TNF-α, but not IL-6, was elevated in the majority of patients with leukemia and in one third of those with solid tumors. These levels rapidly decline following institution of therapy and probably reflect a host response to tumor challenge. IL-6 was elevated (≥ 50 pg/ml) in 74% of children with fever and proven bacteremia. Furthermore, elevated IL-6 levels were found in 81% of children with fever of unknown origin, in whom the levels of C reactive protein exceeded 50 μg/ml on any of the first three days of infection. Presence of neutropenia did not influence IL-6 levels. Thus IL-6 measurement may be a useful tool in the evaluation of fever in children with malignant disease.

April 1997

Persistence of polyomavirus versus immunity to polyoma tumors

ZSOFIA BERKE

Department of Immunology, Microbiology, Pathology and Infectious Diseases, Division of Clinical Virology, Karolinska Institute, Huddinge University Hospital, S-141 86 Huddinge, Sweden

The aim of this thesis was to understand the role of different specific immune effectors with regard to polyomavirus persistence and polyomavirus induced tumor development. Furthermore, an MHC class I restricted short viral peptide was identified as a target for polyomavirus specific recognition of polyoma tumor cells.

Persistence of polyomavirus was followed with a polyoma specific polymerase chain reaction (PCR) in immunocompetent and immunodeficient mice. Persistent polyomavirus DNA could not be detected in normal adult infected mice, in contrast to that observed in normal newborn mice. Persistence of polyomavirus in newborn mice and later tumor development were suggested to be due to that newborn mice do not have a fully matured immune system. In order to specifically analyze the importance of different immune effectors with regard to polyoma persistence, polyomavirus infection was followed in mice with various immune deficiencies. In adult severe combined immune deficiency (SCID) mice, lacking both functional T-, and B-cells, an extensive spread of polyomavirus was observed and the mice succumbed to the infection. In adult CD4-/- or CD8-/- single knockout mice with only one T-cell population, infection with polyomavirus was limited and cleared around 1 month p.i. in most of the animals. However, in CD4-/-8-/- double knockout mice lacking both T-cell

populations a more extensive viral spread was observed, and a persistent polyoma infection was established. Among neonatally infected mice, a proportion of normal, CD4-/- or CD8-/- single knockout and CD4-/-8-/- double knockout mice harbored persistent polyoma DNA several weeks p.i. The frequency of polyoma tumor development was, however, significantly higher in CD4-/-8-/- double knockout mice (29%) compared to CD4-/- or CD8-/- single knockout mice (2% and 11% respectively) and normal mice (6%). Polyomavirus persistence was also followed in adult mice deficient in antibody production. In adult X-linked immune deficiency (XID) mice, with a decreased B-cell count, polyomavirus persistence was established in approximately one third of the mice. IgM-/- single knockout and IgM-/-CD8-/- double knockout mice harbored persistent polyomavirus in most organs 6–12 weeks p.i.

MHC class I restricted peptides were eluted from the surface of a polyoma positive tumor and a short peptide was identified as possible tumor specific transplantation antigen (TSTA). The peptide was a nonamer corresponding to amino acid (aa) sequence 578–586 of polyoma Large T-antigen (LT). In vivo tumor rejection tests showed that a shorter version of this peptide corresponding to LT 578–586 was the most effective as an immunogen.

April 1997

Genetic studies in familial breast cancer

MORAIMA ZELADA HEDMAN

Department of Molecular Medicine, Karolinska Institute, Stockholm, Sweden

This study focuses on familial breast cancer, where some alteration is supposed to be inherited and predispose the individuals to breast cancer. Even when the majority of breast cancer cases are sporadic, a familial breast cancer study might light on over certain genes involved in the ethiology of the disease.

A cohort of 236 families from the Stockholm region is involved in this thesis. The families represented 129 families with hereditary breast or breast-ovarian cancer, 80 families with two close relatives with breast cancer and 27 families with at least 1 case of breast cancer and two or more cases with other types of cancer. Fifty-two of the 236 families were selected from a population of all existing breast cancer patients in Stockholm in a study done 1988–89. The remaining families were recruited through the Cancer Family Clinic at the Karolinska Hospital during 1990–1995.

Loss of heterozygosity (LOH) study on chromosome 17q in tumors from breast-ovarian cancer families showed few losses in the BRCA1 region.

Three different regions of LOH could be distinguished on 17q in familial tumors. No strong correlation was found with lymph node metastases.

Mutation screening of TP53 gene in 109 patients revealed no germline mutations, and few somatic mutations in 51 tumors.

Mutation screening of the BRCA1 gene resulted in a lower frequency of BRCA1 mutations than expected (33% of breast-ovarian cancer families and less than 1% of the site specific breast cancer families).

The alteration in codon 160 of the estrogen receptor gene (ESR) which has been reported in British and Norwegian breast cancer families is not present in this cohort of families. The 999del5 mutation described in the BRCA2 gene, in the Icelandic population could not be found in these families either.

Other genes besides BRCA1, BRCA2 and TP53 are likely to be segregating in breast cancer families in the Stockholm region.

May 1997

Contrast medium enhanced magnetic resonance imaging in diagnosis of breast diseases

BEATA BONÉ

Department of Diagnostic Radiology, Huddinge University Hospital, KARO Institution, Karolinska Institute, Stockholm, Sweden

Contrast medium enhanced magnetic resonance tomography (CME-MRI) is a new imaging method in diagnosis of breast diseases. This method was evaluated in 238 patients (250 breasts) scheduled for breast surgery because of a breast lesion. The sensitivity and specificity of the method was determined. The results were correlated with the histopathological findings. They were also compared to the sensitivity and specificity of X-ray mammography in breast lesions. In the further evaluation, CME-MRI was tested in the assessment of local recurrence of breast cancer in 83 patients who had undergone mastectomy and been reconstructed with a breast implant. Finally, contrast medium enhancement at CME-MRI examination in 50 malignant breast tumours was compared to morphological factors of prognostic importance in order to see if there was any relationship between them. Other morphometric variables were also analysed in both malignant and benign lesions, in order to find out why carcinomas as well as benign breast lesions can be both enhancing and non-enhancing lesions.

The results indicate that CME-MRI of the breast is a highly sensitive method for the detection of breast cancer. However, absence of contrast medium enhancement, or delayed enhancement did not exclude malignancy. Furthermore, some features of benign lesions were associated with contrast medium enhancement which lead to false positive findings, resulting in a rather low specificity of CME-MRI. When mammography and CME-MRI were used together they seemed to be complementary and a very high sensitivity (99%) was achieved. CME-MRI was effective in revealing mammographically occult or equivocal lesions and multifocal tumours, even in dense breasts, but it was less reliable for some invasive lobular cancers, non-invasive ductal carcinomas, fibroadenomas, and hyperplastic breast changes. CME-MRI was superior to physical examination and mammography in detecting local recurrences in patients with breast implants. Nevertheless, we advocate that all three methods, palpation, mammography and CME-MRI are necessary for detecting recurrence at an early stage during the postoperative follow-up.

The contrast medium enhancement in breast cancers at CME-MRI correlated to tumour angiogenesis and proliferating cellular activity. Furthermore, there was a correlation between contrast medium enhancement and tumour malignancy grade as well as tumour invasiveness. These observations suggest that the contrast medium enhancement was influenced by these factors which are considered to be of prognostic value.

In conclusion the contrast medium enhancement at CME-MRI apparently related to the proliferating activity of hyperplastic or neoplastic parenchymal cells and correlated inversely with the interstitial area in carcinomas as well as in benign tumours and non-neoplastic lesions of the breast. These findings support the hypothesis that both these morphometric variables play an important role in the general mechanism of contrast medium enhancement in breast lesions at CME-MRI and also explain the overlap in enhancement pattern between malignant and benign lesions.

May 1997

Molecular genetics of lymphoid malignancies

MATS MERUP

Department of Medicine, Karolinska Institute at Huddinge Hospital, 141 86 Huddinge and Radiumhemmet, Karolinska Hospital, 171 76 Stockholm, Sweden

Advances in molecular genetics during the last decade has made it possible to identify genetic lesions in malignant cells that are specific for disease entities with a common clinical presentation and prognosis.

In chronic lymphocytic leukemia (CLL) deletions in 13q14 are the most frequently occurring abnormalities and deletions cluster around marker D13S319 suggesting that a tumor suppressor gene is located in this region. Southern blot identifies deletions of D13S319 in more than 40% of CLL patients. Interphase FISH is equally efficient in detecting deletions and it also reveals that different subclones can occur with variable numbers of alleles in the D13S319 region.

Trisomy 12 is the most common cytogenetic abnormality in CLL, but it is still unknown how this abnormality contributes to disease development or progression. In a detailed FISH analysis of a case with a chromosome 12 abnormality we found amplification of the 12q13–15 region. The MDM2 gene was found to be most frequently amplified, suggesting that genes in this region can provide a growth advantage for CLL cells.

Deletions of the long arm of chromosome 6 are frequently found in lymphoid malignancies. PCR analysis of loss of heterozygosity (LOH) revealed that deletions of 6q occur in 36% of acute lymphoblastic leukemia (ALL) cases, showing that this abnormality is more frequent than has been previously recognized. A minimal deleted region if 4 cM around marker D6S283 has been identified and our results suggest that this is the location of a tumor suppressor gene relevant for ALL. Our results also indicate that there is at least one more region on 6q that is commonly deleted in NHL that might contain a gene of interest for the development of high grade lymphomas. In B-CLL patients, deletions of 6q are found particularly in a subgroup of patients with aberrant, non-productive rearrangements of the genes for the β chain of the T-cell receptor. Aberrant TcR β gene rearrangement is a rare event in B-CLL occurring in 6% of patients. The reason for the connection between TcR β gene rearrangement and deletion of 6q is not clear.

The BCL-2 protein, which can contribute to disease development and acquisition of resistance to drug therapy, is overexpressed in several different lymphoid malignancies including CLL. However, translocation of the BCL-2 gene seems to be a rare event occurring in 9% of the CLL cases. These results suggest that BCL-2 overexpression in CLL in most cases is caused by mechanism other than gene translocation.

Cytogenetic studies suggest the presence of a candidate gene relevant for development of hairy cell leukemia (HCL) at chromosome 5q13.3. We have defined a YAC clone spanning the breakpoint and two cosmid clones on either side of the breakpoint. Using the cosmids for interphase FISH analysis in HCL patients we detect breakpoints in subclones of the malignant cells. The defined region is closely connected to a recently reported breakpoint region found in AML and MDS, suggesting that the candidate gene in HCL may be identical to a gene that is deleted in myeloid malignancies.

May 1997

Anti-idiotypic immunity in multiple myeloma and monoclonal gammopathy of undetermined significance

SUSANNE BERGENBRANT GLAS

Department of Medicine, Division of Hematology and Infectious Diseases, Karolinska Institute at Karolinska Hospital, Stockholm, Sweden

Monoclonal gammopathies such as multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) are lymphoproliferative B cell diseases. B cells/plasma cells produce the monoclonal immunoglobulin (Ig) which can be detected in plasma and/or urine. Such Ig have unique variable regions of the heavy and light chains and may be regarded as tumour-specific antigens and as such potential targets for immune regulation of the tumour cell clone, by anti-idiotype (Id) immune reactions.

The production of anti-Id Ab against the M-component by primary B cell lines of Epstein-Barr virus transformed PBMC was analysed in patients with MM and MGUS. Patients with advanced disease (MM stage III) had a low and patients with MM stage I and MGUS a high production of such anti-Id Ab ($p < 0.01$).

Anti-Id B cells were studied in patients with MM, MGUS and in healthy controls using an ELISPOT assay. All patients had B cells producing Ab to $F(ab')_2$ fragments of autologous and allogeneic M-components. There was no difference between patients with MM and MGUS with regard to the number of B cells producing Ab against autologous M-component. MGUS and MM patients had autoreactive B cells more frequently than alloreactive B cells ($p < 0.001$). The number of alloreactive B cells did not differ between patients and controls.

T cells reactive with $F(ab')_2$ fragments of autologous and allogeneic M-components were analysed in patients with MM, MGUS and in healthy controls using an ELISPOT assay. In patients, the number of T cells that were stimulated by autologous M-component was higher ($p < 0.01$), than the number of cells activated by allogeneic Ig.

Subpopulations of Id-reactive T cells were defined by demonstrating Th cells with different cytokine-secreting patterns after stimulation by $F(ab')_2$ fragments of autologous M-components using ELISPOT assays. Th1-type cells (secreting IFN-γ and IL-2) were more frequent in patients with a low tumour burden. Th2-type cells (secreting IL-4) dominated in patients with MM stage II–III. The T cell response was MHC class II-restricted. Id-reactive T cells were also demonstrated in MM and MGUS patients with ^{3}H-thymidine-incorporation. The proliferation of cells was higher when stimulating PBMC with $F(ab')_2$ fragments of autologous M-component ($p < 0.05$) than with allogeneic M-components.

Injection of autologous M-component suspended in alum into five MM patients induced an in vitro Id-specific response in both T and B cells. The response was reduced during repeated immunisations.

In conclusion, the results indicate a presence of immunity against Id determinants on monoclonal Ig. Ab, B and T cells with reactivity against $F(ab')_2$ fragments of autologous M-components exist in both MM and MGUS. Their presence in these disorders may indicate a regulatory function. These findings form a basis for further studies aiming at identifying new ways to enhance a specific anti-tumour response in MM.

May 1997