

## ABSTRACTS

**Abstracts of a symposium on "IGFs and Cancer" held at the Martin-Luther-University Halle-Wittenberg, Halle, Germany 15-17 September 2000**

**Abstract 1. Different expression patterns of insulin-like growth factor binding proteins in human pituitary adenomas and craniopharyngiomas**

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Insulin-like growth factors (IGFs) are important growth promoting proteins. Their effects are mediated by IGF receptors, which are involved in various biochemical pathways in the cell and are expected to be a potential target for new therapeutic approaches in cancer. IGF binding proteins (IGFBPs) act as potent modulators of the cellular responses to IGF-I. The presence and distribution of IGFBPs in pituitary tumours has not been explored previously. This study investigates the expression of IGFBPs in human pituitary adenomas and craniopharyngiomas.

Blocks of paraffin wax embedded, formalin fixed tumour tissue from 98 pituitary adenomas, including 27 recurrent tumours, and from 14 primary craniopharyngiomas were selected for detection of IGFBPs 1-6 with rabbit polyclonal antibodies. Immunohistochemistry was performed using the avidin-biotin complex method with 3,3'-diaminobenzidine as chromogenic substance under specific pretreatments. Material from carcinoma in situ of the testis was used as control tissue. The expression of IGFBPs was evaluated semiquantitatively, and the subcellular localisation of the staining was noted.

All tumours expressed different IGFBP types with a variable and diffuse intracytoplasmic staining pattern, except for four craniopharyngiomas and one primary pituitary adenoma, which showed no expression of IGFBP-2. An intracytoplasmic dot-like pattern of expression was found in 10 pituitary adenomas stained with anti-IGFBP-2 antibody, in 106 cases (including 11 craniopharyngiomas) with anti-IGFBP-3 antibody, in 69 tumours (including only three craniopharyngiomas) with anti-IGFBP-4 antibody, in eight pituitary adenomas stained with anti-IGFBP-5 antibody, and in 51 neoplasms (including three craniopharyngiomas) stained with anti-IGFBP-6 antibody. Most tumours displayed an IGFBP staining pattern consisting of a few small cytoplasmic dots. On the other hand, most of the pituitary adenomas (n = 79) and one craniopharyngioma stained with anti-IGFBP-3 antibody revealed many large intracytoplasmic dots. There was a significant difference in the paranuclear dot-like immunostaining for IGFBP-3 between craniopharyngiomas and pituitary adenomas (p < 0.001, Fischer's exact test), but no significant difference in IGFBP expression between primary and recurrent pituitary adenomas.

Human pituitary adenomas and craniopharyngiomas express IGFBPs in different patterns. The role of the cytoplasmic dot-like expression of IGFBP-3 in pituitary adenomas

should be explored more closely. Further investigations are necessary to define the possible prognostic value of the different expression patterns of IGFBP in these tumours.

**Abstract 2. IGF-II inhibits butyrate induced apoptosis of LIM 2405 human colon cancer cells**

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Butyrate is a short chain fatty acid that is formed by bacterial fermentation of fibre in the colon. It has been shown previously to induce apoptosis and inhibit proliferation of colon cancer cells. However, butyrate is a more potent inhibitor of tumorigenesis in vitro than in vivo.

LIM 2405 cells derive from a poorly differentiated colon carcinoma. Sodium butyrate induced apoptosis of these cells is assessed morphologically using Hoechst 33258 DNA staining, and also by DNA laddering and cell death enzyme linked immunosorbent assay (ELISA). When these cells undergo apoptosis, they detach and float in the medium. The effect of butyrate was dose dependent (0-30 mM).

Insulin like growth factor II (IGF-II) is an autocrine growth factor for many colon cancer cells. Pretreatment of LIM 2405 cells with IGF-II (100 ng/ml, one hour) followed by incubation with butyrate (10 mM) + IGF-II for 24 hours increased the mean attached cell number from 69 ± 3% to 95 ± 7% of control (p < 0.01). There was a concomitant decrease in the number of floating (apoptotic) cells from 180 ± 14% to 42 ± 8% of control (p < 0.001). This result was confirmed by decreased intensity of DNA ladders following incubation with IGF-II and by cell death ELISA, which showed that the number of apoptotic cells following treatment with IGF-II + butyrate was approximately four times lower than with butyrate alone (p < 0.001).

Multiple pathways have been implicated in the actions of butyrate. The most important of these is thought to be the inhibition of histone deacetylase. Trichostatin A (TSA) is a specific inhibitor of this enzyme and it had a similar pro-apoptotic effect to butyrate on LIM 2405 cells. Incubation with IGF-II slightly increased attached cell number from 64 ± 5% to 73 ± 6% of control but substantially decreased floating cell number from 233 ± 15% to 49 ± 7% of control (p < 0.001). The inhibition of TSA induced apoptosis by IGF-II was confirmed by cell death ELISA, which showed that the number of apoptotic cells following treatment with IGF-II + TSA was approximately three times lower than with TSA alone (p < 0.01).

Butyrate also has tumorigenic effects such as increasing cell migration. This property was confirmed for LIM 2405 cells (142 ± 29% of control, p < 0.01). IGF-II also increased migration by 80 ± 29% (p < 0.01). In contrast to the effects on apoptosis, incubation of IGF-II and butyrate had an additive effect on migration (261 ± 47%,

p < 0.05 v butyrate). TSA (1 µM) did not increase migration (101 ± 8% of control), suggesting that this effect of butyrate is not dependent on the inhibition of histone deacetylase.

These results suggest that: (1) IGF-II specifically antagonises the antitumorigenic apoptotic action of butyrate; (2) IGF-II acts downstream of histone hyperacetylation. IGF-II expression may be one mechanism whereby colon cancer cells acquire resistance to the antitumorigenic effects of butyrate and may explain at least in part the relatively modest effects of butyrate on tumorigenesis in vivo.

**Abstract 3. The IGF-I receptor in cancer: transformation versus differentiation**

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In most cell types in culture, the insulin-like growth factor receptor (IGF-IR) sends an unambiguous mitogenic signal. In other cell types (such as myoblasts, osteoblasts, adipocytes, oligodendrocytes, neurons, and haemopoietic cells), IGF-I and IGF-II can stimulate either proliferation or differentiation, or both. We have studied the response of haemopoietic cells to IGF-I in 32D cells. 32D cells are murine haemopoietic cells of the myeloid lineage, which undergo apoptosis within 24 hours after withdrawal of interleukin 3 (IL-3). When 32D cells overexpress (even modestly) the IGF-IR, they survive in the absence of IL-3 and, with the addition of IGF-I, they actually grow for about 48 hours. Then, the cells begin to differentiate along the granulocytic pathway, and eventually decrease in number, as one would expect from terminally differentiated cells. 32D cells do not express IRS-1 or IRS-2. If 32D IGF-IR cells are stably transfected with IRS-1 cDNA, to generate 32D IGF-IR/IRS1 cells, the cells no longer differentiate, they grow indefinitely in the absence of IL-3, and they form tumours in animals. Conversely, if 32D cells are transfected with a plasmid expressing Shc proteins they rapidly differentiate. Thus, at least in the case of 32D cells and the IGF-IR, the "cell context" is the availability of individual substrates of the IGF-IR. The presence or absence of a single transducing molecule makes the difference between malignant transformation and terminal differentiation, based on differences in the signalling pathways of the IGF-IR.

**Abstract 4. Signalling pathways involved in antiproliferative effects of IGFBP-3**

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The proliferation of cell populations represents the balance between cell division and cell death. Insulin-like growth factor binding protein 3 (IGFBP-3) has the potential to modulate both of these processes, either by

affecting the interactions of IGFs with the type 1 IGF receptor (IGF-1R), or by alternative IGF-1R independent mechanisms. The tumour suppressor protein p53 could initiate some IGFBP-3 mediated effects, because IGFBP-3 gene expression is activated by p53 in response to DNA damaging stimuli such as ionising radiation (IR). Cell death (apoptosis) may be induced by an alteration in the ratio of pro-apoptotic and anti-apoptotic proteins of the bcl-2 family—for example, the bax : bcl-2 ratio. In T47D breast cancer cells, which lack wild-type p53 and are relatively resistant to IR, transfection with IGFBP-3 cDNA causes an increase in apoptosis as determined by nuclear fragmentation or TUNEL labelling. In clonogenic survival assays, IGFBP-3 transfected cells have a significantly reduced survival rate compared with controls, when exposed to increasing doses of IR, and indices of apoptosis are significantly increased above that seen in control cells. Thus, IGFBP-3 is able to sensitise the IR resistant cell line to radiation induced apoptosis even in the absence of wild-type p53. The mechanism appears to involve a post-transcriptional increase in bax, because levels of Bax protein but not mRNA are increased by IGFBP-3 and further by IR. Anti-apoptotic Bcl-x<sub>L</sub> levels are correspondingly decreased (T47D cells do not express Bcl-2). Evidence is accumulating that IGFBP-3 signalling involves its nuclear translocation. We previously used mutagenesis to implicate the basic domain residues of IGFBP-3 (228–232), KGRKR, in this process. More recently we have shown, in addition, the importance of basic residues 215–216 and 220–222 in the nuclear localisation sequence (Schedlich *et al*, *J Biol Chem* 2000;275:23462). Nuclear import of IGFBP-3 is an energy dependent process and is blocked by antibodies against importin-β; furthermore, direct interaction of IGFBP-3 with importin-β can be shown by western blot. Permeabilisation experiments indicate that IGFBP-3 is retained in the nucleus, but the nuclear elements with which it interacts, and other proteins which it might co-transport to the nucleus, are unknown. IGFBP-3 signalling is also known to be affected by the ERK/mitogen activated kinase (MAP) kinase pathway, because we have shown that breast cancer cell lines expressing oncogenic ras (for example, MCF-10T, Hs578T) are relatively IGFBP-3 resistant and can be sensitised to its inhibitory effect by the MEK inhibitor PD98059. MCF-10T cells remain sensitive to transforming growth factor β (TGF-β) inhibition despite their ras overexpression, but in other cell lines this is reported to block TGF-β signalling via a MAP kinase mediated mechanism. We showed previously that sensitivity to exogenous IGFBP-3 in T47D cells requires an active TGF-β signalling pathway, because added IGFBP-3 was ineffective in these cells (which lack the TGF-β receptor type II and are normally TGF-β resistant) unless they were transfected to overexpress the TGF-β receptor, and exogenous TGF-β was added (Fanayan *et al*, *Growth Hormone and Insulin-like Growth Hormone Research* 1999;9:338). These data suggest that MAP kinase might modulate cellular IGFBP-3 sensitivity via interaction with TGF-β signalling intermediates. The exact pathways involved and the reason why T47D cells are differentially responsive to exogenous compared with transfected IGFBP-3 remain to be resolved.

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**Abstract 5. Effectiveness of the phosphatidylinositol-3 kinase inhibitor LY294002 against growth, survival, and motility of Ewing's sarcoma cells**

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Given that several lines of evidence indicate that the insulin-like growth factor I receptor (IGF-1R) is implicated in the autocrine and paracrine control of Ewing's sarcoma growth and may be particularly important in the pathogenesis of this tumour, the main objective of this study is to provide a basis for the development of future treatment strategies targeting IGF-1R in this neoplasm. Previously, we reported that IGF-I promotes growth, survival, and migration of Ewing's sarcoma cells. IGF-I has been shown to modulate transformation, cell growth, and survival through different, although partially overlapping, signal transduction pathways. In particular, the anti-apoptotic effect of IGF-1R as well as its chemotactic action appears to be mainly dependent on the activation of phosphatidylinositol-3 kinase (PI3-K). In this study, we evaluated the effects of LY294002, a potent inhibitor of PI3-K, on the proliferation, survival, and migration of Ewing's sarcoma cells. Incubation with LY294002 (1, 5, and 10 μM) induced a significant inhibition of Ewing's sarcoma cell growth in vitro. This effect was dose dependent and partially reversed by the addition of exogenous IGF-I (10–100 ng/ml). A consistent reduction of the S-phase rate and a significant induction of apoptosis was observed after LY294002 treatment. Moreover, LY294002 was able to affect the migratory ability of Ewing's sarcoma cells. Finally, we found that the inhibition of PI3-K by LY294002 rendered these cells more sensitive to the chemotherapeutic agent doxorubicin. In conclusion, these findings suggest an important role of PI3-K inhibitors as a potentially useful treatment for Ewing's sarcoma patients.

**Abstract 6. Expression of IGF-1, IGF-1R, and IGFBP-3 in eight sarcoma cell lines**

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Insulin-like growth factor 1 (IGF-1) is among the most prevalent growth factors secreted by skeletal cells that are considered autocrine regulators of osteoblastic cell function. IGF-1 has mitogenic activity for bone cells and enhances the differential function of the osteoblast. The IGF-1 receptor (IGF-1R) is a 350 kDa glycoprotein. The cytoplasmic protein tyrosine kinase is activated by ligand binding to the extracellular domains of the receptor. Skeletal cells synthesise six of the known IGF binding proteins (IGFBPs), although the pattern of their expression varies with the cell line studied and culture conditions used. Overexpression of IGF-1R or its ligand IGF-1 is frequently associated with abnormal growth, cellular transformation, and the inhibition of apoptosis in tumour cells. The aim of this study was to characterise the expression of IGF-1, IGF-

1R, and IGFBP-3 in eight sarcoma cell lines as basic data for the further study of redifferentiation and dedifferentiation mechanisms in human sarcomas.

Four human osteosarcoma cell lines (SAOS-2, MG 63, SKOS, and HOS), two Ewing's sarcoma cell lines (MHH-ES1, ESP 38), and two human chondrosarcoma cell lines (SW1353, HST 819T) were cultured and grown on coverslips for direct immunostaining and microscopy. For reverse transcription polymerase chain reaction (RT-PCR) analysis total RNA was isolated from cell cultures using a commercially available kit. Coverslips were prepared for immunostaining using commercially available polyclonal antibodies. To study cell proliferation MTT was also done for each cell line 24 hours, 48 hours, and 72 hours after culturing of 5000 cells.

Transcripts of IGF-1, IGF-1R, and IGFBP-3 were detected in all osteosarcomas as well as in both Ewing's sarcoma cell lines and correlated well with the immunoreactivities. In both chondrosarcoma cell lines the expression of IGF-1 mRNA was lower than was seen in the osteosarcoma and Ewing's sarcoma cell lines, whereas IGF-1R and IGFBP-3 transcripts were adequately expressed. However, both chondrosarcoma cell lines had lower levels of IGF-1, IGF-1R, and IGF-BP3 immunoreactivity. MTT test evaluation revealed that the chondrosarcoma cell lines had a lower proliferation rate when compared with other sarcoma cell lines. Our data demonstrated that overexpression of the IGF-1/IGF-1R system is associated with abnormal growth in human sarcoma cells.

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**Abstract 7. Serum IGF-I and TGF-β1 in patients with early stages of small cell lung cancer (SCLC), non small cell lung cancer (NSCLC), and breast cancer**

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Conflicting data suggest that transforming growth factor β1 (TGF-β1) can inhibit or promote the progression of neoplastic cells. It seems that in early stages of cancer the neoplastic cells are sensitive to TGF-β1 mediated growth arrest so that TGF-β1 can act as an antitumour promoter. However, in advanced stages of cancer, resistance to such TGF-β1 action develops. In addition, neoplastic cells produce large amounts of TGF-β1, which may enhance tumour invasion and metastasis by intensifying angiogenesis, immunosuppression of the whole organism, and locally by enhancing the formation of extracellular matrix. On the other hand, IGF-I probably takes part in the promotion of the growth of normal and neoplastic cells and plays a role in the transformation processes, in angiogenesis, and neoplastic progression. It is thought that TGF-β1 is one of the cytokines that can antagonise the stimulatory effect that IGF-I has on the proliferation of neoplastic cells.

The aim of the work was to: (1) assess the serum concentrations of TGF- $\beta$ 1 and IGF-I in patients with early stages of small cell lung cancer (SCLC; 16 males), non-small cell lung cancer (NSCLC; 13 males), and breast cancer (15 premenopausal women); and (2) determine correlations between TGF- $\beta$ 1 and IGF-I in these patients.

TGF- $\beta$ 1 was determined by enzyme linked immunosorbent assay (ELISA) and IGF-I was estimated by means of radioimmunoassay (RIA).

In patients with early stages of SCLC and NSCLC, serum TGF- $\beta$ 1 concentrations were significantly higher in comparison with the control group (healthy subjects). IGF-I concentrations in the patients did not differ significantly from the control group. Serum TGF- $\beta$ 1 and IGF-I concentrations in premenopausal patients with breast cancer did not differ significantly from those of healthy women.

No interaction between mean TGF- $\beta$ 1 and IGF-I was shown in any of the studied groups.

**Abstract 8. Cloning and characterisation of a longer fragment containing a breakpoint downstream of the IGF2 gene that is associated with overexpression of IGF2 mRNA in colorectal tumours**

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The insulin-like growth factors (IGFs) control cell proliferation, differentiation, and death. IGF2 mRNA and peptide overexpression was associated with tumour progression in different experimental systems. We have reported IGF2 mRNA overexpression in 30% of primary human colon cancers (Lambert *et al*, *Int J Cancer* 1991;48:826–30). The colon cancers contained increased concentrations of the corresponding peptide (Lambert *et al*, *Int J Cancer* 1991;48:826–30, 1991, Winkler *et al*, *Horm Metabol Res* 1999;31:148–54). Interestingly, in two tumours overexpressing very high levels of the transcript, the genes showed structural alterations of their 3' region. A fragment containing the breakpoint was cloned by the vectorette PCR technique and characterised (Hodzic *et al*, *Oncogene* 1999;18:4710–17).

Here, we report the isolation of a DNA fragment containing the breakpoint from a genomic library constructed from the DNA of one of the tumours containing the rearrangement. The library was screened using a PCR based method described by Israel *et al*, *Nucleic Acids Res* 1993;21:2627–31. We isolated and characterised an 11 kb hybrid fragment, containing 4 kb of the normal IGF2 gene and 7 kb of the new sequence fused to the 3' region of the IGF2 gene. A restriction map of the fragment was constructed. This fragment will be used to identify the origin of the new sequence and to understand how and if it influences IGF2 gene expression.

**Abstract 9. A Functional IGF-1 receptor–green fluorescent protein chimera to study kinase regulation by tyrosine phosphatases**

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A chimera of the insulin-like growth factor 1 receptor (IGF-1R) and green fluorescent protein (GFP) has been engineered by fusing GFP to the C-terminus of IGF-1R. Upon transient or stable transfection into IGF-1R null fibroblasts the IGF-1R–GFP protein becomes localised to the plasma membrane, becomes autophosphorylated in response to IGF-1, and is capable of phosphorylating endogenous substrates. Downstream signalling molecules such as AKT and ERKs are activated in response to this receptor at levels comparable with wild-type IGF-1R. Immunofluorescence with anti-phosphotyrosine antibodies using TRITC for detection shows an increase in cellular tyrosine phosphorylation in response to IGF-1, some of which colocalised with the IGF-1R–GFP.

We then used the IGF-1R–GFP to investigate further the physiological importance of negative regulators of IGF-1R kinase activity that were identified in a *Schizosaccharomyces pombe* yeast screen developed in our laboratory. We found that coexpression of the protein tyrosine phosphatase PTP-1B (a known negative regulator of insulin receptor kinase activity) with the  $\beta$ -chain of IGF-1R dramatically inhibits autophosphorylation and abolishes IGF-1R kinase activity. IGF-1 stimulated Cos cells co-expressing IGF-1R–GFP and PTP-1B have reduced tyrosine phosphorylation on both IGF-1R–GFP and cytoplasmic proteins, as detected by anti-phosphotyrosine immunofluorescence. These data indicate that IGF-1R–GFP is a useful tool with which to analyse IGF-1R function, and further demonstrate that PTP-1B is a negative regulator of the IGF-1R. This study also shows that a fluorescence based assay can be used to study potential negative regulators of the IGF-1R kinase.

**Abstract 10. IGF status is altered by isoflavone supplementation in healthy women**

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Isoflavones are oestrogen-like plant compounds that can act as anti-oestrogens and may play a role in coronary heart disease and breast cancer aetiology. Isoflavones may also, like Tamoxifen, alter insulin-like growth factor (IGF) status, thus potentially reducing the risk of breast cancer.

The aim of this study was to assess the effect of one month of isoflavone supplementation (80 mg/day) on IGF status in healthy premenopausal (n = 16) and postmenopausal (n = 7) women in a randomised, placebo controlled crossover study with a minimum two month washout period. Fasting blood samples were collected at baseline and at 28

days for the postmenopausal subjects, and at baseline, day 1–3, day 6–8, day 12–15, day 21–23, and day 26–28 for the premenopausal subjects to assess the effects of the menstrual cycle on IGF status. Concentrations of IGF-1, IGF binding protein 1 (IGFBP-1), and IGFBP-3 were measured by an enzyme linked immunosorbent assay (ELISA) kit from Diagnostic Systems Laboratories. For postmenopausal subjects, IGF-1 and IGFBP-3 concentrations were not affected by isoflavone supplementation. However, IGFBP-1 concentrations were significantly increased (mean, 44.3 (SE, 3.4) v mean, 54.7 (SE, 4.1) ng/ml; p < 0.05; baseline v post-supplement). For premenopausal subjects, there was a significant increase in both IGF-1 over the menstrual cycle (mean, 169.6 (SE, 12.9) v 192.1 (SE, 12.6) ng/ml; p < 0.05; baseline v post-supplement), and IGFBP-3 concentrations (mean, 3116 (SE, 99) v 3319 (SE, 111) ng/ml; p < 0.05; day 1–3 v day 21–23) when subjects were on placebo, whereas no significant increase was seen in these subjects while on the isoflavone supplement (IGF-1: mean, 198.4 (SE, 18.3) v mean, 204.2 (SE, 11.5) ng/ml; p = NS; IGFBP-3: mean, 3275 (SE, 157) v 3343 (SE, 112) ng/ml; p = NS; day 1–3 v day 21–23).

This study shows that one month of isoflavone supplementation can alter IGF status in both premenopausal and postmenopausal women, which may suggest a role for these dietary compounds in breast cancer prevention.

**Abstract 11. IGF status is altered by Tamoxifen in breast cancer patients**

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Increased concentrations of insulin-like growth factor 1 (IGF-1) have been found in patients with breast cancer compared with healthy controls, and healthy subjects with high IGF-1 concentrations have been shown to have an increased risk of developing breast cancer. Tamoxifen is thought to reduce IGF-1 concentrations initially and to increase concentrations of its binding proteins.

The aim of this study was to compare IGF-1, IGF binding protein 1 (IGFBP-1), and IGFBP-3 concentrations in patients with breast cancer (n = 7) and control subjects (n = 14), and to assess the effect of Tamoxifen on IGF status in these patients. Non-fasting blood samples were collected from patients with breast cancer before surgery and after at least five months of Tamoxifen treatment, and from age matched healthy control subjects. Concentrations of IGF-1, IGFBP-1, and IGFBP-3 were measured using an enzyme linked immunosorbent assay (ELISA) kit from Diagnostic Systems Laboratories. IGF-1 and IGFBP-3 were not significantly different in cases and controls. IGFBP-1 was significantly lower in cases than in controls (mean, 9.39 (SE, 1.23) v mean, 21.8 (SE, 5.5) ng/ml; p < 0.01). Tamoxifen treatment had no significant effect on IGF-1 concentrations, but increased IGFBP-1 (mean, 9.39 (SE, 1.23) v mean, 54.3 (SE, 26.3) ng/ml; p < 0.05; pre-Tamoxifen v post-Tamoxifen) and IGFBP-3 concentrations (mean, 3139 (SE, 164) v

mean, 3651 (SE, 196) ng/ml;  $p < 0.05$ ; pre-Tamoxifen *v* post-Tamoxifen).

This study shows that patients with breast cancer have reduced concentrations of IGFBP-1 compared with healthy control subjects. It also shows that although Tamoxifen had no effect on IGF-1 concentrations, it can increase concentrations of both IGFBP-1 and IGFBP-3, thus altering the overall concentration of bioavailable IGF-1 in the circulation. This may be another mechanism whereby Tamoxifen exerts its anticarcinogenic effect in the secondary prevention of breast cancer.

#### Abstract 12. IGF binding proteins: independent regulators of cancer cell growth—basic aspects and clinical significance

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Serum insulin-like growth factor binding protein 3 (IGFBP-3) has been shown to be a risk modulator for the development of prostate, breast, and colon cancers. IGFBP-3 can decrease cancer risk by counteracting the effects of IGF-I, but may also act directly on cells. Multiple IGF independent actions of IGFBPs on cancer cells have been identified in recent years. These include growth inhibition, apoptosis, and the regulation of gene expression. Evidence for cell surface receptors for IGFBPs exists, but actual membrane IGFBP receptors have yet to be cloned. Interactions of IGFBPs with integrins and matrix proteins are known to modulate cell function and signal transduction through a variety of pathways. IGFBPs also interact directly with multiple extracellular molecules with known growth regulatory functions ranging from heparin to transferrin. To date, it is entirely unknown how some IGFBPs enter cells, yet several IGFBPs, including IGFBP-2, IGFBP-3, and IGFBP-5 have been detected intracellularly. Once inside the cell, IGFBPs appear to be able to enter the nucleus, compatible with the presence of a nuclear localisation sequence (NLS) that allows IGFBP-3 and IGFBP-5 to bind  $\beta$ -importin, which serves as a nuclear shuttle. Recently, the human papillomavirus oncoprotein E7 was found to bind IGFBP-3 intracellularly and to facilitate its proteosomal degradation, which leads to the loss of its apoptotic functions and its nuclear localisation. The roles of nuclear IGFBPs appear to include transcriptional regulation of cell cycle and apoptosis related genes. The main nuclear receptor for IGFBP-3 appears to be the retinoid receptor-X (RXR). Yeast two hybrid experiments, co-immunoprecipitation, GST pull down experiments, ligand blots, and confocal microscopy confirm specific high affinity binding of IGFBP-3 to RXR in the nucleus. IGFBP-3 enhances RXR mediated signalling, but inhibits the signalling of the RXR partners RAR and PPAR. Furthermore, RXR knock-out cells lose their response to IGFBP-3. In cell line models of cancer, IGFBP-3 and RXR ligands have additive effects on the induction of apoptosis. The roles of other IGFBPs in the nucleus are under investigation. Together, these lines of research lead to a clearer picture of IGFBPs as nuclear molecules, which probably serve as coactivators

and corepressors of traditional and/or orphan nuclear receptors. The importance of these IGFBP functions in cancer, where nuclear receptors play a key role, is being evaluated.

#### Abstract 13. Serum IGFs and prostate cancer

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Insulin-like growth factor I (IGF-I) and IGF-II are peptide hormones known to cause cell proliferation and to inhibit apoptosis. They have both been implicated in the development of prostate cancer. A family of six proteins, the IGF binding proteins (IGFBPs), tightly bind to IGF-I and II. IGFBP-3 binds to 95% of circulating IGF-I and IGF-II, controlling the levels maintained in the circulation. It is thought that while the IGFs promote prostate cell growth, the IGFBPs inhibit it. Interestingly, prostate specific antigen (PSA), a marker for prostate cancer, is also known to be a specific IGFBP-3 protease. Recent studies have shown that serum IGF-I may be raised in patients with prostate cancer. The objective of this study was to investigate whether serum concentrations of IGF-I, IGF-II, and IGFBP-3 are different in patients found to have prostate cancer compared with those with no evidence of cancer following prostate biopsies.

One hundred and two consecutive patients with suspected prostate cancer as a result of either a raised PSA ( $> 4$  ng/ml) or an abnormal digital rectal examination went on to have transrectal ultrasound guided prostate biopsies. Before the biopsies, blood samples were taken for analysis of IGF-I, IGF-II, IGFBP-3, and PSA. The biopsies were examined to distinguish those with prostate cancer from those with no evidence of malignancy.

Sixty three (61.7%) patients had no evidence of malignancy (mean age, 67.2; range, 47–87 years), and 39 (38.2%) were found to have prostate cancer (mean age, 72.6; range, 53–91 years). Table 1 shows the results of the IGF-II, IGF-I, IGFBP-3, and PSA analyses in relation to the presence or absence of cancer in these patients.

Thus, serum concentrations of IGF-I and IGF-II were significantly raised in patients found to have prostate cancer compared with those with no evidence of prostate cancer. It was also found that IGFBP-3 was raised in those found to have prostate cancer.

Table 1 Abstract 13. IGF-I, IGF-II, IGFBP-3, and PSA values in patients with and without cancer

| Values (ng/ml)           | No cancer               | Cancer                  | Logistic regression accounting for age |
|--------------------------|-------------------------|-------------------------|--|
| Mean PSA (SD; range)     | 8.2 (9.5; 0.1–60.7)     | 100.9 (268.9; 1.6–1630) | $p < 0.001$                            |
| Mean IGF-I (SD; range)   | 120.8 (39.6; 53–217)    | 135.0 (43.4; 61–245)    | $p = 0.025$                            |
| Mean IGF-II (SD; range)  | 722.2 (193.3; 297–1136) | 798.6 (230.6; 462–1563) | $p = 0.049$                            |
| Mean IGFBP-3 (SD; range) | 4.97 (1.49; 1.9–9.3)    | 5.54 (1.75; 3.7–12.8)   | $p = 0.026$                            |

IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; PSA, prostate specific antigen.

#### Abstract 14. IGFs and IGFBP-3 and IGFBP-5 in germ cells, carcinoma in situ (CIS), and seminoma of the human testis

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Seminomas are common malignant germ cell tumours in men aged between 30 and 50 years. Current knowledge supposes carcinoma in situ (CIS) of the testis to be the precursor of both seminomas and non-seminomas.

Insulin-like growth factor I (IGF-I) and IGF-II and their binding proteins: IGFBPs 1–6, a highly conserved family of proteins that bind IGFs with great affinity, are known to play an important role in the growth and differentiation of many tissues and probably in the promotion of cancer development. The aim of this study was to investigate whether IGF-I, IGF-II, and some of their binding proteins have any relevance in the development of testicular germ cell tumours.

Therefore, the pattern of IGF-I, IGF-II, and IGFBP-3 and IGFBP-5 expression in normal testicular tissue, early tumour stages (CIS and early invasive interstitial tumour cells (ITCs)), and solid seminomas of the human testis was investigated by means of enzyme immunohistochemistry.

IGF-I immunoreactivity was found in a proportion of the spermatogonia: approximately 50% of all CIS and ITC cells and in 60% of seminoma cells. Four of 20 cases lacked IGF-I immunoreactivity in tumour cells.

IGF-II immunoreactivity was not found in spermatogonia (weak immunostaining was detectable in some spermatocytes and spermatids). CIS cells were also negative for IGF-II. Weak IGF immunoreactivity could be seen one of 13 cases of ITC. In 12 of 20 cases a weak positive staining for IGF-II was found in seminoma cells.

IGFBP-3 was detected in part of the nuclei of spermatogonia, nuclei and cytoplasm of spermatocytes and spermatids, and in part of the cytoplasm and very few nuclei of the CIS cells (weak staining). It could not be detected in ITC, but was found in 11 of 20 cases in seminoma cells (only in the periphery of the tumour nodes).

IGFBP-5 immunoreactivity was found in the cytoplasm and 20% of the nuclei of spermatogonia, the cytoplasm of spermatocytes, and the cytoplasm and 10% of the nuclei of spermatids. Strong immunostaining was detected in all CIS cells (mostly cytoplasmic) and ITC (mostly cytoplasmic). IGFBP-5 was found in the cytoplasm of 70% of seminoma cells.

In conclusion, IGF-I immunoreactivity is found in normal germ cells as well as tumour

cells. In contrast, IGF-II can only be detected in cells of solid seminoma and never in CIS cells. This could indicate that IGF-II plays a role in the development of solid germ cell tumours, which is not required in early tumour stages. IGFBP-3 and IGFBP-5 are found both in normal germ cells and tumour cells of all stages, although the quality of this reaction changes. In normal cells nuclei and (partly) cytoplasm are stained, whereas in tumour cells immunoreactivity is found only in the cytoplasm and not in the nuclei. The relevance of these findings is still unclear but it is likely that the IGF/IGFBP system plays a role in the development of testicular germ cell tumours. However, further speculations about the (patho-)physiological role of these findings cannot be made by means of immunohistochemistry. Functional studies to investigate these findings are required.

**Abstract 15. Signal transduction of IGFBP-2 through integrins possibly affects survival and metastasis of tumours**

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Recently, it has become apparent that the insulin-like growth factor binding protein 2 (IGFBP-2) plays a role in the tumorigenesis and growth of human tumours. In addition to direct effects on the biological actions of the IGFs, IGF independent actions through binding to cell surface integrins via the RGD amino acid motif were expected, as has been shown for IGFBP-1.

Hence, we studied the cellular binding and signalling of IGFBP-2, and its effects on cell attachment and proliferation in a Ewing sarcoma (A673) cell line expressing high amounts of IGFBP-2 and a breast cancer cell line (Hs578T) expressing no IGFBP-2. <sup>125</sup>I-IGFBP-2 bound to both cell lines and binding was competed for by IGFBP-1 and synthetic RGD peptide, but not by other IGFBPs or RGE peptide. Binding of IGFBP-2 was found to be specific to the  $\alpha 5 \beta 1$  integrin, whose presence was confirmed by immunoblot and flow cytometry. Furthermore, binding induced significant dephosphorylation (up to 40%) of the focal adhesion kinase (FAK), an important protein of the intracellular signalling of the integrin. Because we detected IGFBP-2 dependent alterations in growth and cell adhesion of the tumour cells, IGFBP-2 may play a role in the survival and dissemination of these tumours. Most probably, IGF independent actions of IGFBP-2 through integrin signalling are involved in these processes.

**Abstract 16. Analysis of IGF-I and IGF-II analogue binding to the type 1 receptor and correlation to ability to inhibit apoptosis**

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Insulin-like growth factors (IGFs) play an important role in the development of cancers because they promote survival by activating anti-apoptotic signalling pathways as a result of binding type 1 receptors. In this study, we have compared binding of IGF-I, IGF-II, and various IGF analogues to a recombinant high affinity type 1 receptor following BI-Acore analysis. The IGF-I analogues have been designed either with mutated residues that result in lower binding affinities for the type 1 receptor or IGF binding proteins (IGFBPs). Concurrent investigations evaluated the effects of IGFs and the analogues in the PC12 cell model of serum deprivation induced apoptosis. Levels of apoptosis were quantitated by counting the number of condensed or fragmented Hoechst 33258 stained nuclei. IGF-I at 1 nM completely prevented apoptosis in PC12 cells. Overall, these studies support the conclusion that type 1 receptor binding affinity correlates directly with the ability of these analogues to prevent serum deprivation induced PC12 cell apoptosis.

**Abstract 17. IGFs protect human colon carcinoma cells from IFN- $\gamma$ /TNF- $\alpha$  induced apoptosis independently of cell-matrix and cell-cell interactions via NF- $\kappa$ B activation**

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We recently reported that insulin-like growth factors (IGFs) and insulin conferred to HT29-D4 human colon cancer cells an almost complete resistance to apoptosis induced by tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) after sensitisation of the cells by interferon  $\gamma$  (IFN- $\gamma$ ) or a combination of anti-Fas antibody and TNF- $\alpha$ .

Here, we investigate the ability of des-(1-3)-IGF-I (dIGF) to protect HT29-D4 cells from IFN- $\gamma$ /TNF- $\alpha$  induced apoptosis in the absence of cell-extracellular matrix (ECM) and cell-cell interactions. For this purpose, the cells were seeded in serum free medium on plates coated with poly-L-lysine (PLL), the polycationic nature of which prevents integrin engagement, and polyhydroxyethylmethacrylate (polyHEMA), which denies cells attachment to ECM. In addition, function blocking antibodies were added to the cell cultures, either a mixture of monoclonal antibodies against  $\alpha_5$ ,  $\alpha_6$ , and  $\beta 1$  integrin subunits, which recover the pattern of HT29-D4 cell expressed integrins, or the MB2 anti-E-cadherin monoclonal antibody. Under all these culture conditions, cells remained sensitive to apoptosis induced by IFN- $\gamma$ /TNF- $\alpha$ , and dIGF continued to protect the cells from death. Thus, IGFs can mediate an anti-apoptotic effect totally independently of integrin and E-cadherin mediated signalling.

We have confirmed the activation by dIGF of several of the previously reported signalling events, especially tyrosine phosphorylation of IRS-1 and activation of phosphatidylinositol 3-kinase (PI3-K)/AKT, and p38 and ERK mitogen activated protein kinase (MAP) pathways. However, inhibitors of PI3-K

(LY294002 and wortmannin), p38 (SB203580), and MEK/ERK (PD098059) had no effect on the dIGF mediated anti-apoptotic effect. Moreover, dIGF was able to suppress apoptosis regardless of whether it potentiated tyrosine phosphorylation of FAK (a major transducer of integrin mediated survival), as in ECM adherent cells, or not, as in anchorage independent cells. Thus, IGFs do not use these canonical survival pathways to convey the anti-apoptotic signalling in colonic cancer cells.

In contrast, pharmacological agents inhibiting the NF- $\kappa$ B pathway were able to reverse the dIGF anti-apoptotic effect totally. These agents included inhibitors of the phosphorylation (BAY11-7082; aspirin) and degradation (lactacystin) of I $\kappa$ B, and NF- $\kappa$ B transactivation (triptolide). Accordingly, dIGF strongly potentiated TNF- $\alpha$  induced production of interleukin 8 (IL-8), a proinflammatory chemokine, which requires activation of NF- $\kappa$ B for its expression. Thus, we conclude that the potentiation of TNF- $\alpha$  induced NF- $\kappa$ B dependent survival/inflammatory signalling constitutes the major pathway through which IGFs prevent the destruction of colonic cancer cells by death factors, such as those of the TNF superfamily. dIGF did not increase nuclear levels of NF- $\kappa$ B in TNF- $\alpha$  stimulated cells even though NF- $\kappa$ B dependent IL-8 production was greatly increased. This suggests that dIGF might act by regulating the transcriptional activity of NF- $\kappa$ B either by enhancing its phosphorylation by a yet unknown kinase and/or its association with a cofactor in the nucleus.

These findings may have important biological and clinical implications. Alterations of IGF-II bioavailability have been described in colon cancer cells in relation to the plasmin mediated proteolysis of IGFBPs. It follows that upregulation of bioavailable IGF-II by increasing resistance to cytokine induced apoptosis in the colon cancer cells, and especially in those that detach from ECM in the process of metastasis, should contribute to the limited efficiency of both immune and conventional anticancer treatments. Thus, the IGF axis may constitute a novel attractive therapeutic target to improve the cell death inducing weapons that the immune system uses for combating cancer.

**Abstract 18. Overexpression of IGFBP-2 enhances the tumorigenic potential in adrenocortical tumour cells: identification of target genes**

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Under normal conditions, insulin-like growth factor binding protein 2 (IGFBP-2) is a negative growth factor (Hoefflich *et al. Endocrinology* 1999;140:5488-96), which probably acts by interfering with the binding of IGF to its receptor. Such negative interference had been described in IGF dependent 293 cells and in various IGF dependent colon carcinoma cells Höflich, *et al. FEBS Lett* 1998;434:329-34). IGFBP-2 in these cell systems resulted in an inhibition of cell proliferation, which could be completely compensated for by the addition

of exogenous IGF-I or IGF-II. However, it is known from various malignant cellular systems that IGFBP-2 is upregulated at the mRNA and protein level (for example, adrenocortical tumours). Moreover, it has been shown that IGFBP-2 serum concentrations correlate positively with the malignant features of these tumours. To investigate the consequences of enhanced IGFBP-2 expression we have produced stable IGFBP-2 overexpressing clones of adrenocortical tumour cells (Y-1 cells). Interestingly, overexpression of IGFBP-2 in Y-1 tumour cells resulted in a dramatic increase of malignant growth and an altered cellular morphology by IGF independent mechanisms (Hoeflich, *et al. Cancer Res* 2000;60:834-8). Here we have used our IGFBP-2 overexpression models (transfected Y-1 and 293 clones) to search for genes regulated by IGFBP-2 and investigated whether these genes might represent potential targets responsible for aberrant cell proliferation within multistep events of tumour development and growth. Screens were performed using cDNA arrays followed by RT-real time PCR quantification of candidate gene expression relative to the expression of housekeeping genes using SybrGreen™ as the fluorescent dye. Genes induced in both models encode proliferation associated proteins, tumour markers, and genes potentially involved in stress adaptation or multidrug resistance syndrome. The results thus clearly support the concept of IGFBP-2 as a malignant cofactor. As one of the genes induced by IGFBP-2 overexpression, glutathione S-transferase (GST) displayed a strong induction of gene expression in both models. Inhibition of GST by use of its specific inhibitor ethacrynic acid in micromolar concentrations led to absolute cellular mortality after five days of incubation. Moreover, coincubation of ethacrynic acid and glutathione in its reduced form enhanced the cytotoxic effect of ethacrynic acid, suggesting specificity of the cytotoxic effect. We conclude that GST is indispensable for Y-1 tumour cells and current efforts are directed to the question of whether enhanced GST concentrations might lead to aberrant cell proliferation by conferring resistance to oxidative or toxic stress. From the general pattern of genes induced in two independent models of IGFBP-2 overexpression we conclude that, despite its negative function in IGF dependent growth, IGFBP-2 may be a malignant cofactor that can induce distinct proliferation associated and/or stress adaptive genes.

**Abstract 19. Potential mechanism for IGFBP-3 induced apoptosis in an oesophageal carcinoma cell line**

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In addition to modulating insulin-like growth factor (IGF) action, IGF binding protein 3 (IGFBP-3) has been shown to exert IGF independent actions and to participate actively in apoptotic pathways. While it has been shown that IGFBP-3 can independently induce apoptosis in certain cell lines, we have shown IGFBP-3 to enhance inducers of apoptosis in the Hs587T breast carcinoma cell line and in the KYSE 190 oesophageal carcinoma cell line.

Because the other high affinity IGFBPs do not elicit these effects in these cell lines, it is hypothesised that the variable mid region of IGFBP-3 and, in particular, the serine residues may be important in eliciting the specific effects of IGFBP-3. The aim of this study was therefore to examine whether the variable mid region of IGFBP-3 was responsible for its independent effects on enhancing apoptosis in cancer. IGFBP-3 was pre-incubated with ABESF (serine protease inhibitor) or phosphorylated with casein kinase II. The KYSE 190 cell line, plated at  $5 \times 10^5$  cells/well, was transferred to serum free medium for 24 hours before dosing with either IGFBP-3, IGFBP-3-ABESF complex, or phosphorylated IGFBP-3 for a further 24 hours. The conditioned medium was removed and the cells washed with phosphate buffered saline (PBS). The cells were triggered to cell death with  $10 \text{ J/m}^2$  UV irradiation. Serum free media was replaced for a further 24 hours and cell death measured by trypan blue exclusion. Apoptosis was confirmed by the presence of a pre-G1 peak on flow cytometry. IGFBP-3 alone had no effect on cell death over 24 or 48 hours, but significantly enhanced UV induced cell death by 23% ( $p = 0.03$ ) at 24 hours. Pre-incubation with either IGFBP-3-ABESF or phosphorylated IGFBP-3 negated its enhancing effects on UV induced cell death. To confirm the specificity of these effects cells were incubated with a 15 amino acid peptide spanning the serine residues of the mid terminal, and this peptide, like intact IGFBP-3, was again capable of enhancing UV induced apoptosis. Manipulation of the serine sites within the variable mid region of IGFBP-3 indicates that the phosphoserine residues are crucial for its apoptotic action, and confirm the fundamental IGF independent, pro-apoptotic effects of IGFBP-3 in cancer.

**Abstract 20. The IGF receptor I in human B cell malignancies**

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Multiple myeloma (MM) and B cell chronic lymphocytic leukemia (B-CLL) are two types of human B lymphocyte malignancy. Whereas MM is characterised by the accumulation of malignant plasma cells that maintain proliferative potential, B-CLL is characterised by the accumulation of CD5 expressing B cells that exhibit a low proliferative rate but high levels of apoptosis resistance. Because the type I insulin-like growth factor receptor (IGF-IR) is often overexpressed in human malignancies and plays key roles in both growth control and apoptotic resistance of tumour cells, we began a coordinated study of the role of this receptor in both of these diseases. With respect to MM, although interleukin 6 (IL-6) has been identified as a major growth factor in this disease, the precise mechanism by which IL-6 stimulates MM cell proliferation remains unknown. We have demonstrated that IGF-I may play a key role in IL-6 mediated MM cell growth. Thus, we have observed that IGF-I alone may stimulate MM cell growth as well as significantly augment IL-6 stimulated cell growth. These results suggest that the IGF

response is a crucial component of IL-6 driven growth of myeloma cells. In an extension of this work, we have also assessed IGF-IR levels on our panel of IL-6 dependent MM cell lines, normal human B cells and plasmablasts, and freshly isolated plasma cells from patients with monoclonal gammopathies. Normal B cells and plasmablasts did not express detectable levels of IGF-IR as determined by flow cytometry. By contrast, each of five IL-6 dependent MM cell lines expressed greatly raised levels of the receptor. To determine whether this receptor was also overexpressed on fresh patient plasma cells, we used three colour flow cytometric analysis to assess IGF-IR levels (using the  $\alpha$ IR3 monoclonal antibody) on CD38 high and CD45 low plasma cells. Our results indicate that myeloma cells express IGF-IR levels that are significantly higher than is found on plasma cells from patients with monoclonal gammopathy of undetermined significance. With respect to B-CLL, although peripheral blood leukaemic cells are typically all found in the G0 phase of the cell cycle, they are highly resistant to undergoing apoptosis, suggesting that although the IGF-IR may not transmit a proliferative signal in this disease, it may impact on cell survival. Indeed, in preliminary studies using flow cytometric analysis and RNase protection assays, we have observed that the IGF-IR is overexpressed in the leukaemic clone in the vast majority of the B-CLL samples that we have tested. Efforts are currently under way to dissect further the role of this important receptor in the biology of this disease. Because IGF-IR gene expression is tightly regulated in normal cells, our results demonstrating the deregulation of IGF-IR expression in both myeloma and B-CLL cells suggest a pivotal role for this receptor in the biology of both of these B cell malignancies, as well as the progression of disease.

**Abstract 21. IGF-I and IGFBP-3 in benign prostatic hyperplasia and prostate cancer**

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In view of evidence indicating significant involvement of the insulin-like growth factor (IGF) system in the pathogenesis of prostate cancer, we measured serum IGF-I and IGF binding protein 3 (IGFBP-3) in men with benign prostatic hyperplasia (BPH;  $n = 75$ ) or prostatic carcinoma (CaP;  $n = 84$ ). The age matched patient populations were selected to have circulating prostate specific antigen (PSA), the most reliable predictor of CaP, in the overlapping diagnostic "grey zone" range of  $\sim 4-10 \mu\text{g/litre}$ . Of particular interest was investigation of intact, fragment, and total IGFBP-3 values in relation to PSA, which is also a well established IGFBP-3 protease. Among the key findings were significantly higher IGF-I and intact IGFBP-3 concentrations in CaP compared with BPH ( $p < 0.001$ ), whereas changes in fragment and total IGFBP-3 were not significant. As expected, total PSA values were similar in the two groups of patients ( $p = 0.173$ ), whereas free PSA values were significantly lower in those with CaP ( $p < 0.001$ ). IGF-I and IGFBP-3 (intact and

total) correlated significantly ( $p = 0.024$  to  $< 0.001$ ) and inversely ( $r = -0.26$  to  $-0.35$ ) with free PSA in BPH but not in CaP, and no correlations were found in comparisons involving total PSA. Statistical analysis of the various markers and their combinations indicated superior performance of IGF-I/free PSA (ROC area under the curve; AUC = 0.728) and intact IGFBP-3/free PSA (AUC = 0.737) ratios in discriminating between BPH and CaP compared with the currently used free/total PSA ratio (AUC = 0.689). Multivariate logistic regression models confirmed the observed relations and identified IGF-I/free PSA and intact IGFBP-3/free PSA as independent factors in predicting the presence of CaP. We conclude that increases in IGF-I and intact IGFBP-3 values are positively associated with the presence of CaP in patients with low to moderately raised PSA, and that their measurements in relation to PSA may help improve diagnostic discrimination between BPH and prostate cancer.

#### Abstract 22. IGFFBPs in rare tumours of the ovary in childhood

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Germ cell tumours and tumours of the gonads comprise only 3.2% of these rare solid tumours in childhood. The prediction of the course of these neoplasms is very difficult because of the absence of a reliable prognostic marker. A prognostic effect of the analysis of insulin-like growth factor binding proteins (IGFBPs) of cystic fluid was shown in ovarian tumours from adult women. Therefore, because it might be of value to investigate the presence and distribution of IGFFBPs in some rare tumours of the ovary in childhood, we undertook such a study.

Representative blocks of paraffin wax embedded, formalin fixed tumour tissue from a sex cord stromal tumour with annular tubules (SCTAT), a juvenile granulosa cell tumour (JGCT), a dysgerminoma (DG), and a gonadoblastoma (GB) occurring in the ovaries of infants were studied with rabbit polyclonal antibodies against IGFFBPs 1–6. Immunohistochemistry was performed using the avidin–biotin complex method with 3,3' diaminobenzidine as dye and under specific pretreatments. Material from carcinomas in situ of the testis was used as control tissue. The expression of IGFFBPs was estimated semiquantitatively and the localisation of the immunoreaction was studied.

All tumours expressed the IGFFBPs 2–6 in a various diffuse cytoplasmic staining pattern. IGFBP-1 was found only in a distinct dot-like pattern in JGCT. This cytoplasmic dot-like expression could also be seen with an anti-IGFBP-3 antibody in DG and SCTAT. In addition, a dot-like expression was detected with an anti-IGFBP-5 antibody in JGCT. Nuclear expression of the IGFFBPs was seen in the different tumours. In addition to the expected nuclear staining with anti-IGFBP-3 and anti-IGFBP-5 antibodies, we also found some tumours with nuclear expression of IGFBP-1, IGFBP-4, and IGFBP-6.

In conclusion, SCTAT, JGCT, DG, and GB express IGFFBPs in various patterns. These differences suggest that the expression

of IGFFBPs could be used as a diagnostic tool. It is tempting to speculate about the potential prognostic importance of IGFBP accumulation, but further investigations with a higher number of these tumours and also examining the clinical data are necessary to establish the role of IGFFBPs in prognosis. The unusual cytoplasmic dot-like pattern of IGFBP expression will be a challenging field for prospective morphological investigations.

#### Abstract 23. Transient activation of JNK kinases is required for suppression of apoptosis by the IGF-I receptor

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The insulin-like growth factor receptor (IGF-1R) activated by its ligands IGF-I or IGF-II mediates suppression of apoptosis, which is crucial for tumorigenesis and the survival of many tumour types. Several signalling pathways can be activated by the IGF-1R that lead to the suppression of apoptosis, in different cell types, including those mediated by phosphatidylinositol 3-kinase (PI-3 kinase), AKT, mitogen activated protein kinase (MAP) kinase pathways, and phosphorylation of the Bcl-2 family member Bad. Previous results from a mutational analysis of the receptor indicated that PI-3kinase independent antiapoptotic signals could be propagated from the IGF-1R in lymphocytic cells and fibroblasts. Therefore, we investigated signalling pathways activated or regulated by particular regions of the IGF-1R that may be uniquely involved in the suppression of apoptosis and transformation. In particular, we investigated the role of the stress activated protein kinase pathways (SAPK)/Jun N-terminal kinase (JNKs) and the p38 MAP kinase pathways. JNK and p38 activation can be regulated by IGF-I stimulation of different cell types, but a role for these proteins in IGF-1R mediated antiapoptotic or transformation activity is not known.

The interleukin 3 (IL-3) dependent FL5.12 lymphocytic cells that overexpress the wild-type IGF-1R (WT), which we have previously shown to be protected from IL-3 withdrawal by IGF-I, was used as a model system to measure IGF-I mediated activation of JNK and p38 pathways. We found that IGF-I stimulation of WT cells induced transient activation of JNK and led to robust phosphorylation of c-Jun. p38 activity was not altered in response to IGF-I. Activation of the JNK pathway occurred in the presence of PI-3 kinase inhibitors and was enhanced by anisomycin treatment. The quinone reductase inhibitor, dicoumarol, at concentrations that specifically inhibited JNK activation but not activation of PI-3 kinase or AKT, completely abrogated IGF-1 mediated suppression of apoptosis, but not IL-3 mediated survival of WT cells. Analysis of a series of IGF-1R mutants in these cells demonstrated that activation of JNK did not require the C-terminus of the receptor. These results indicate that JNK activation leading to c-Jun phosphorylation is necessary for the suppression of apoptosis by the IGF-1R.

#### Abstract 24. Effect of all-trans retinoic acid on the expression of IGF-1, IGF-1R and IGFBP-3 in pancreatic tumour cell lines

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Pancreatic cancer is a rapid progressive disease with a mortality of approximately 99%. The pathway downstream of p53 involves this important tumour suppressor gene in insulin-like growth factor 1 (IGF-1) receptor (IGF1-R) bioactivity, either via direct regulation of IGF-1R levels, or modulation of IGFs via transactivation of the IGF binding proteins (IGFBPs). The IGF-1R is a 350 kDa glycoprotein. The cytoplasmic protein tyrosine kinase is activated by ligand binding to the extracellular domains of the receptor. Its ligand, IGF-1, a 70 amino acid peptide, is an important mediator involved in the signal transduction pathway of human growth hormone. IGF-1 bioavailability is downregulated by specific IGFFBPs, especially IGFBP-3. Overexpression of IGF-1R or its ligand IGF-1 is frequently associated with abnormal growth, cellular transformation, and inhibition of apoptosis in tumour cells. Retinoic acid (RA) is an agent with well characterised differentiation inducing properties. The aim of this study was to analyse the effect of all-trans RA on the expression of IGF-1, IGF-1R, and IGFBP-3 in three pancreatic tumour cell lines.

PA-TU 8902 cells were established from primary ductal pancreatic adenocarcinoma (grade II), CAPAN-1 cells from the liver metastasis of a pancreatic ductal adenocarcinoma, and HUP-T3 cells from the malignant ascites of poorly differentiated adenocarcinoma. Confluent cells were stimulated either with solvent or with 1  $\mu$ M RA for 24, 48, and 72 hours. The expression of IGF-1, IGF-1R, and IGFBP-3 mRNA was analysed by reverse transcription polymerase chain reaction (RT-PCR) using specific primers. IGF-1: sense 5'-ATC CTT CTC TCC TCA TTC TTC-3', antisense 5'-GAT ACA CAG ACA CAG ATA AAA G-3'; IGF1-R: sense 5'-TCC ACA TCC TGC TCA TCT CC-3', antisense 5'-AGA AGT CAC GGT CCA CAC AG-3'; and IGFBP-3: sense 5'-TCA GAG CAC AGA TAC CCA G-3', antisense 5'-ACA GCC GCC TAA GTC AC-3'. Commercially available antibodies were used for western blotting analysis.

RA induced increased mRNA expression of IGF-1 and IGFBP-3 in HUP-T3 cells. IGF-1 transcripts were more highly expressed after RA treatment for 24 and 72 hours in CAPAN-1 cells when compared with the control. No change in IGF-1 mRNA values could be detected in PATU 8902 cells. No difference in mRNA expression of IGF-1R and IGFBP-3 after RA treatment could be found in CAPAN-1 and PA-TU 8902 cells. Western blotting analyses confirmed these RT-PCR results. Our data showed that IGF-1, IGF-1R, and IGFBP-3 were variably expressed in pancreatic cell lines. RA has a clear effect on the poorly differentiated cell line HUP-T3.

Our findings suggest that all-trans retinoic acid induces the increased expression of IGF-1 and IGFBP-3 in a poorly differentiated pancreatic carcinoma cell line. The

biological relevance of RA induced IGF-1/IGFBP-3 expression has yet to be clarified.

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#### Abstract 25. IGFBP-2 induced intracellular signalling

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Integrins are transmembrane glycoproteins that exist as heterodimeric complexes of non-covalently associated  $\alpha$  and  $\beta$  subunits. We demonstrated specific binding of insulin-like growth factor binding protein 2 (IGFBP-2) to  $\alpha_5\beta_1$  integrin. Therefore, the influence of IGFBP-2 binding on integrin signalling was investigated.

An important protein of the integrin signalling pathway is the focal adhesion kinase (FAK), the phosphorylation status of which reflects signals transduced by  $\alpha_5\beta_1$  integrin. We established a procedure to measure the FAK phosphorylation level using immunoprecipitation of FAK and investigated the phosphorylation of FAK in relation to IGFBP-2 concentrations and depletion by anti-IGFBP-2 antibodies in two adhesive tumour cell lines: Ewing sarcoma A673 cells and breast tumour Hs578T cells. The influence of IGFBP-2 on cellular proliferation and cell detachment was measured.

IGFBP-2 induced the dephosphorylation of FAK in the Ewing sarcoma (reduced by 30%) and the breast tumour cell lines (reduced by 40%). Dependent on IGFBP-2 concentrations, proliferation is reduced (10%) and the number of detached cells increased (31%).

The results demonstrate that IGFBP-2 is able to induce an intracellular signal that results in reduced cell proliferation. These results agree with the hypothesis that IGFBP-2 plays a role in the biology of solid tumours.

#### Abstract 26. Quo vadis childhood diabetes!

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Both type I and type II diabetes mellitus (DM) are found in childhood. Type I is the more frequent and more serious form between birth and the end of puberty. It is an organ specific autoimmune disease that progressively destroys the pancreatic  $\beta$ -cells until metabolic decompensation masks the clinical disease. Type I DM requires lifelong insulin replacement treatment, and even good metabolic control (achieved in a minority of patients) can only postpone or ameliorate, but not prevent, the late vascular complications. The aetiology of type I DM is a combination of genetic and environmental factors, the latter being blamed for the fast and progressive increase in the incidence of type I DM in affluent countries in the past decades. Specific and sensitive immunological methods enable the detection of the autoimmune process in subjects with a high genetic risk (family members). Immunosuppressive intervention trials using toxic sub-

stances (cyclosporine) have failed; at present, oral insulin and nicotinamide trials are ongoing in selected patients. However, they are applied too late, as is a trial with a heat shock analogue peptide injected at diagnosis. Of importance is increasing laboratory and epidemiological evidence that enteroviruses may be the initiating factor of the autoimmune process in the perinatal period, followed by additional agents found in food (cow's milk nutritional additives) and car produced pollutants, with repeated infections continuing to damage the  $\beta$ -cells until exhaustion.

Identification of the responsible viruses for triggering the disease should enable primary prevention programmes. There is no cure for type I DM—pancreatic cell transplantation and insulin gene therapy are only future dreams—and because the present technological advances in treatment (synthetic insulins, insulin delivery pumps, multidisciplinary team, etc) are of limited value it is imperative to look for means to stop the ever increasing incidence of this lifelong disease, which owing to its numbers and consequences has become a public health problem.

In recent years an increase in obesity has been accompanied also by a rise in the incidence of type II DM (insulin resistance) mainly but not only in genetically susceptible populations. Rarer forms of DM in children are associated with chromosomal or mitochondrial diseases.

#### Abstract 27. IGF-I: a growth hormone

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There is an ongoing controversy about whether the hormone responsible for linear growth is the pituitary growth hormone or insulin-like growth factor I (IGF-I), acting in an independent manner. IGF-I is synthesised by the liver under the control of growth hormone (GH) and released into the circulation, from where it acts in an endocrine manner on organ and somatic growth. IGF-I is also expressed in most tissues of the organism, where it is also regulated by GH but exerts local effects (paracrine action). Laron syndrome (LS; primary GH resistance) caused by molecular defects of the GH receptor or post receptor pathways, which leads to a deficiency of GH dependent IGF-I from conception, is a unique model to study the above dilemma in humans. Recently IGF-I gene knockout (KO) or GH receptor KO mice have enabled the study of animal models.

Since the first description of LS in 1966, we have followed 51 patients, many since infancy. Newborns with LS are slightly shorter (42 to 47 cm) than healthy babies (49–52 cm), suggesting that IGF-I has some influence on intrauterine growth. Newborn IGF-I KO mice are 30% smaller. The postnatal growth rate of patients with LS is very slow, the distance from the lowest normal centile increasing progressively. If untreated, the final height is 100–136 cm in females and 109–138 cm in males. There is also acromicria, organomicria including the brain, heart, gonads, genitalia, and a great retardation in skeletal maturation. The availability of biosynthetic IGF-I since 1988 has enabled its long term administration to

children with LS. In the absence of GH activity, IGF-I induced all the protein anabolic effects of GH. It accelerated linear growth rates, as observed by us and others, to 8–9 cm in the first year of treatment compared with 10–12 cm/year during GH treatment. The growth rate in the following years was 5–6.5 cm. In infancy, the differential growth stimulating effect on linear growth between IGF-I and GH was also evident. In contrast, the catch up growth of the head circumference (brain growth) in young children was similar for both hormones. It thus seems that an optimal linear growth effect of IGF-I depends on the presence of GH. It has been suggested that GH is needed to stimulate the early differentiation of the germinative cartilage cells, an IGF-I independent effect, because IGF-I mRNA is not expressed in these cells. However, IGF-I has a GH independent action on the central nervous and other tissues. Noteworthy are the opposing effects between IGF-I and GH on insulin secretion and Lp(a) synthesis.

In conclusion, IGF-I is an important growth hormone, mediating the pituitary GH protein anabolic and linear growth promoting effect. It also has a GH independent growth stimulating effect, which with respect to cartilage cells is optimised by the synergistic action of GH.

#### Abstract 28. Expression of IGF-1, IGF-1R, and IGFBP-3 in gastric carcinoma

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Gastric carcinoma is the fourth most frequent carcinoma. Its incidence shows great geographical differences. The mortality rate in Germany is 30/100 000 male inhabitants. Earlier studies have shown a relation between abnormal growth and spontaneous tumour formation as well as the overexpression of the insulin-like growth factor receptor (IGF-1R) and its ligand IGF-1. IGF-1/IGF-1R are required for the regulation and stimulation of cell proliferation and also for optimal growth in various cell types. IGF-1 bioavailability is downregulated by specific IGFBPs, especially IGFBP-3. The aim of this study was to analyse the expression of IGF-1, IGF-1R, and IGFBP-3 transcripts in stomach carcinoma.

Total RNA was isolated from 12 gastric carcinomas. For reverse transcription polymerase chain reaction (RT-PCR) analysis specific primers were used (IGF-1: sense 5'-ATC CTT CTC TCC TCA TTC TTC-3', antisense 5'-GAT ACA CAG ACA CAG ATA AAA G-3'; IGF-1R: sense 5'-TCC ACA TCC TGC TCA TCT CC-3', antisense 5'-AGA AGT CAC GGT CCA CAC AG-3'; and IGFBP-3: sense 5'-TCA GAG CAC AGA TAC CCA G-3', antisense 5'-ACA GCC GCC TAA GTC AC-3'). Histological diagnosis and classification were carried out on each specimen.

RT-PCR analysis revealed that IGFBP-3 mRNA was found in all tissues. The IGF-1 transcript was strongly expressed in five of 12 tissues when compared with the positive control. The IGF-1R transcript was highly expressed in two and variably expressed in 10

of 12 carcinoma tissues. Because of the limited number of tissues investigated no correlation between the pTNM and the expression of IGF-1/IGF-1R could be found. Our data showed that IGF-1, IGF-1R, and IGFBP-3 are expressed in human gastric carcinomas.

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#### **Abstract 29. The role of the IGF-I receptor in cell proliferation and apoptosis**

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The insulin-like growth factor I (IGF-I) receptor plays an important role in cellular proliferation and in preventing apoptosis. Although the major signalling pathways, such as the phosphatidylinositol 3-kinase (PI3-kinase) and mitogen activated kinase (MAP) kinase pathways, have been shown to be involved in these important functions, other pathways are also involved.

Oestradiol and IGF-I stimulate MCF-7 breast cancer derived cells in a synergistic manner, at the level of early signalling events including activation of the IGF-I receptor, IRS-1 and PI3-kinase. In addition, synergism is seen at events that include cell cycle components such as the cyclins and the cyclin dependent kinase (CDK) inhibitors. Thus, with regard to cellular proliferation, signalling pathways emanating from the IGF-I receptor and the oestrogen receptor may interact at multiple levels.

IGF-I can protect cells against UV radiation damage. Using 4-NQO, a UV mimetic agent, NIH-3T3 cells DNA are damaged, as demonstrated by the comet assay as well as a failure to proliferate, with cell cycle arrest. Stimulation of these cells with IGF-I overcomes the DNA damage and the cell cycle arrest; cells once again enter S phase.

The major signalling pathway involved in IGF-I receptor rescue of DNA damage under these circumstance is the p38 MAP kinase pathway, as demonstrated by the inhibition of IGF-I rescue in the presence of SB202190, a specific inhibitor of p38 $\alpha$  and  $\beta$ . Furthermore, IGF-I receptor activation repairs UV irradiated adenovirus infected into NIH-3T3 cells; again suggesting an effect of the IGF system on DNA repair.

In vivo, the role of circulating IGF-I in tumour progression was studied. Administration of recombinant human IGF-I to mice shortened the latency period of appearance of tumours and stimulated more rapid tumour growth. Reduction of the circulating levels of IGF-I, on the other hand, delayed the onset of tumours. Thus, the IGF/IGF-I receptor system is involved in tumour growth.

#### **Abstract 30. Lycopene interferes in vivo and in vitro with the IGF system**

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The incidence of prostate and breast cancer in the Western world has been increasing at an accelerating pace. To fight this trend, recent research has focused on the role of diet

and related lifestyle exposure in disease prevention. Recently, it was found that the insulin-like growth factor I (IGF-I) blood concentration is a powerful risk factor for prostatic and breast cancer (similar to the way that cholesterol level predicts the risk of heart disease). The same research group also reported that the intake of lycopene reduces prostate cancer risk. In view of these results, our work was focused on two questions: (1) Can lycopene supplementation reduce IGF-I blood values? (2) Does lycopene inhibit IGF activity in cancer cells?

We measured the absorption of dietary lycopene and the effect of this supplementation on IGF-I blood concentration in patients undergoing elective haemorrhoidectomy. Tomato lycopene oleoresin (given orally 15 mg twice/day) or a placebo were administered for three to four weeks before surgery. The lycopene concentration and isomer distribution in the blood and in the surgically removed tissues were measured by high performance liquid chromatography (HPLC). Lycopene was found to increase after supplementation from  $0.31 \pm 0.021$  to  $0.56 \pm 0.053$  nmol/ml. In 15 of the 28 patients in the lycopene treated group, the IGF-I blood concentration decreased more than 10% after supplementation. In the placebo treated group IGF-I values in 20 of 28 patients did not change. These preliminary results should be examined further in other healthy subjects and in patients with cancer.

Lycopene interference with IGF-I activity in vitro was studied with human cancer cell lines. Growth stimulation of MCF-7 mammary cancer cells by IGF-I was noted to be greatly reduced by physiological concentrations of lycopene (0.75  $\mu$ M), while growth of unstimulated cells was inhibited only at higher concentrations of the carotenoid (3  $\mu$ M). We found that lycopene treatment greatly reduced IGF-I stimulation of both tyrosine phosphorylation of insulin receptor substrate-1 and binding capacity of the AP-1 transcription complex. These effects were not associated with changes in the number or affinity of IGF-I receptors, but rather with an increase in membrane associated IGF binding proteins. This suppression was associated with inhibition of IGF stimulated cell cycle progression, from G1 to S phase, of serum starved (synchronised) cells. IGF-I stimulation of the control cells resulted in a two to fourfold increase in cyclin D1 mRNA and protein values. The pRb protein level and the ratio of the inactive phosphorylated form to the active hypophosphorylated form increased. These changes were significantly attenuated by lycopene treatment, whereas the protein concentrations of CDK2, CDK4, cyclin E, and the CDK inhibitor p27 did not change. However, the activity of CDK2 was reduced. Cyclin D expression is greatly increased in breast cancer biopsies. Its transcription is mediated, in part, by AP-1 proteins. Therefore, we used a plasmid carrying a luciferase reporter gene driven by a 1745 bp fragment from the cyclin D promoter that contains the functional AP-1 site. It was found that both retinoic acid and lycopene decreased IGF-I induced upregulation of AP-1 activity in this assay.

In summary, we conclude that lycopene and other carotenoids interfere with the IGF-I system at several levels.

#### **Abstract 31. Identification of new IGF-1 receptor interacting proteins using the yeast two hybrid system**

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Signalling by the insulin-like growth factor 1 receptor (IGF-1R) plays a crucial role in cellular growth, differentiation, and survival. Various signalling substrates and targets that bind to the IGF-1R have been described. However, none of these transducers is unique to the IGF-1R and thus could be exclusively responsible for the unique biological features of the IGF-1R compared with other receptor tyrosine kinases, including the insulin receptor.

We used a modified version of the yeast two hybrid system to identify new cytosolic IGF-1R binding proteins. The intracellular domain of the  $\beta$ -subunit of the IGF-1R was fused to the dimer forming LexA DNA binding domain, which stimulates the activation of the wild-type receptor and induces inter-chain phosphorylation of the receptor domains on tyrosine residues.

In addition to known substrates of the IGF-1 receptor, such as p85 PI3K and Grb10, we isolated several proteins that have not been described previously as IGF-1R ligands. Two of the new IGF-1R interacting proteins (IIPs) show binding specificity for the IGF-1R and do not interact with the insulin receptor: IIP-1 is a 36 kDa phosphoprotein, which contains a PDZ domain. Binding of IIP-1 to the IGF-1R is independent of kinase activity. IIP-10 is a novel 26 kDa protein with an unknown domain structure. Interaction of IIP-10 with the IGF-1R requires activation of the IGF-1R tyrosine kinase.

This indicates that specific targets of the IGF-1R exist that might trigger survival and counteract apoptosis and thus might represent interesting pharmaceutical targets for anti-cancer treatment.

#### **Abstract 32. Different patterns of regulation of IGF-II and IGFBP-3 gene expression by inducing differentiation of adrenocortical carcinoma H295R cells**

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Adrenal masses are among the most common tumours in humans. Highly expressed insulin-like growth factor II (IGF-II) is implicated in the development of adrenocortical carcinomas. IGF bioactivity is modified by IGF binding proteins (IGFBPs). To shed more light on the role of the IGF system in the development of adrenocortical carcinomas, we studied the regulation of IGF-II and IGFBP-3 genes in the cultured human adrenocortical carcinoma NCI-H295R cell line.

In cultured H295R cells, IGF-II is abundantly expressed at the mRNA level, whereas IGFBP-3 mRNA is hardly detectable with northern blotting. Induction of steroidogenesis related gene expression (including steroidogenic acute regulatory protein, low and

high density lipoprotein receptors, and cholesterol side chain cleavage enzyme) and cortisol secretion by (Bu)2cAMP treatment was associated with a reduction (up to 60%) in IGF-II but an increase (up to 300%) of IGFBP-3 mRNA accumulation. Both effects of (Bu)2cAMP on IGF-II and IGFBP-3 levels are dose dependent. However, although the reduction of IGF-II gene expression appeared only after long term incubation (longer than 48 hours), the induction of IGFBP-3 mRNA accumulation was detectable within two hours of treatment. The increase of IGFBP-3 was associated with the upregulation of c-myc and c-fos proto-oncogene expression. Two site immunoradiometric assay for IGFBP-3 demonstrated that changes in IGFBP-3 concentrations in the conditioned media paralleled cellular IGFBP-3 mRNA levels. In addition, long term treatment with (Bu)2cAMP (4–7 days) inhibited cell proliferation (as much as 40% of the control), as analysed by incorporation of bromodeoxyuridine.

In summary, the different patterns of regulation of IGF-II and IGFBP-3 gene expression in H295R cells suggest that they may have opposite roles in the differentiation and proliferation of adrenocortical carcinoma cells.

#### **Abstract 33. IGF-I antisense and triple helix strategies in cellular gene therapy of tumours**

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Insulin-like growth factor I (IGF-I) is a 70 amino acid polypeptide involved in cell and tissue differentiation. IGF-I expressing PCC-3 and PCC4 embryonal carcinomas cells, derived from mouse teratocarcinoma, are widely used as an in vitro model system to study the regulation of cell determination and differentiation, especially in cells of neuroglial origin. The antisense strategy showed that tumour cells when transfected with the vectors encoding IGF-I suppression lost tumorigenicity, became immunogenic, and induced a tumour specific immune response involving CD8+ lymphocytes. Recently, a triple helix approach for IGF-I gene therapy of glioma was described. Our objective was to establish the parameters of cells used for IGF-I triple helix strategy. PCC-3 embryonal carcinoma cells derived from murine teratocarcinoma expressing IGF-I were used as a model. The cells transfected with vectors inducing IGF-I RNA–DNA structures (triple helixes) stopped producing IGF-I, changed phenotype, expressed MHC-I and B-7 molecules, and also showed the feature of apoptotic cells (at least 60% of cells). The IGF-I “triple helix” transfected cells injected subcutaneously into syngeneic 129 mice lost tumorigenicity. The same triple helix cells when co-transfected in vitro with vectors encoding both MHC-I and B-7 antisense cDNA stopped expressing MHC-I and B-7; moreover, the number of apoptotic cells significantly diminished. The injection of these double co-transfected cells into 129 Sv mice has resulted in the development of the teratocarcinoma tumour. Comparatively used IGF-I antisense strategy confirmed these observations. The results demonstrated that IGF-I antisense or triple helix trans-

fectured cultured cells simultaneously show immunogenic and apoptotic characteristics, and that there is a relation between these two cell processes.

The relation between the two phenomena, immunogenicity and apoptosis, is crucial to understanding the mechanism of IGF-I triple helix gene therapy. Moreover, this point is important for the selection of cell clones used for the treatment of human cancers expressing IGF-I; the described observations show that IGF-I triple helix cells should express both MHC-I and B-7 molecules, and be apoptotic (60% of cultured cells). The data obtained using the mouse teratocarcinoma model resemble the data that we obtained recently with human cancer cells. The establishment in our laboratories of transfected IGF-I triple helix cell lines derived from human glioma and hepatoma patients constitutes the beginning of preclinical studies concerning triple helix cellular therapy of tumours.

#### **Abstract 34. Age related SD scores of serum levels of IGF-I, IGF-II, IGFBP-2, IGFBP-3, and acid labile subunit in 67 paediatric tumours**

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Tumorigenesis and malignant transformation of many tumour types are associated with disorders at different regulatory levels of the insulin-like growth factor (IGF) system. In addition to increased production of IGFs and the IGF-I receptor (IGF-IR), high affinity IGF binding proteins, in particular IGFBP-2 and IGFBP-3, can actively affect tumour growth and metastasis. Alterations in IGFBP serum concentrations are therefore considered as diagnostic parameters indicating a malignant tumour or, in turn, for monitoring tumour treatments. So far, however, the role of the IGFBPs in paediatric neuroblastoma and sarcomas is poorly understood.

Hence, we measured IGF-I, IGF-II, IGFBP-2, IGFBP-3, and acid labile subunit (ALS) serum values in 47 paediatric sarcomas (11 peripheral neuroectodermal tumour (PNET), nine embryonal rhabdomyosarcoma (RMS), eight alveolar RMS, seven osteosarcoma, 12 others) and in 20 neuroblastomas by radioimmunoassays (RIAs) and enzyme linked immunosorbent assay (ELISA) and calculated the SD scores on the basis of age dependent in house reference ranges.

We found strongly raised (up to 6 SD) IGFBP-3 concentrations in Ewing sarcoma, PNET and osteosarcoma, as compared with healthy children, whereas IGFBP-2, IGF-I, and IGF-II were significantly decreased. In contrast, in neuroblastoma IGFBP-2 was raised, whereas IGFBP-3 and the IGFs were decreased. Interestingly, there was an overall negative correlation between IGFBP-2 and IGFBP-3 serum concentrations. In conclusion, IGFBP-2 and IGFBP-3 serum concentrations may be of value in the diagnosis of malignant paediatric sarcomas and neuroblastomas.

#### **Abstract 35. IGFBPs modulate integrin signalling independently of IGF-I in Hs578T human breast cancer cells**

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We have demonstrated previously that although insulin-like growth factor binding proteins (IGFBPs) 1–6 alone have no significant effects on cell growth of Hs578T human breast cancer cells, they have differential effects on apoptotic signalling pathways. Whereas IGFBP-1, IGFBP-2, and IGFBP-6 had no effect, IGFBP-3 could significantly enhance, whereas IGFBP-4 and IGFBP-5 could dramatically inhibit, ceramide (C2) induced apoptosis. These effects of the IGFBPs were independent of IGF-I. We have also demonstrated in IGF responsive human breast cancer cells that the mitogenic effects of IGF-I were completely negated in the presence of a non-apoptotic dose of a synthetic RGD containing peptide (RGD). These data indicate that crosstalk occurs between the IGF family and integrin signalling pathways.

Our aims were to investigate whether the differential effects of IGFBPs 1–6 on apoptosis in Hs578T human breast cancer cells were occurring via interaction with integrin signalling pathways.

Cells grown in vitro were either: (1) treated with or without IGFBP-3 (100 ng/ml) for 30 minutes, followed by immunoprecipitation of whole cell lysates with anti-phosphotyrosine and western immunoblotting with anti-focal adhesion kinase (FAK); or (2) treated with or without IGFBPs (100 ng/ml) for one hour in suspension before plating on ECM gel for 30 minutes. Relative cell adhesion was assessed by cell counting.

After 30 minutes exposure to IGFBP-3 there was a significant 36% decrease in FAK phosphorylation in whole cell lysates. In addition, the adhesion of suspensions of Hs578T cells on to ECM gel over 30 minutes was unaffected by IGFBPs 1 and 6, inhibited by IGFBP-3 (30%;  $p < 0.01$ ) and RGD at 10  $\mu$ g/ml (24%;  $p < 0.001$ ), but significantly accentuated in the presence of either IGFBP-4 (10%;  $p < 0.05$ ) or IGFBP-5 (24%;  $p < 0.001$ ), following incubation with IGFBPs for one hour, compared with control cells in the absence of IGFBPs.

These data suggest that IGFBPs 3, 4, and 5 independently of IGF-I may have the ability to interact with integrin signalling to modulate adhesion and in doing so prime the cells and thereby influence their subsequent response to apoptotic stimuli.

#### **Abstract 36. Differential effects of TGF- $\beta$ on IGFBP-3 production in normal versus cancerous breast epithelial cells**

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Transforming growth factor  $\beta$  (TGF- $\beta$ ) is a pluripotent cytokine capable of stimulating or inhibiting cell growth depending on the target cell. TGF- $\beta$  stimulates insulin-like growth factor binding protein 3 (IGFBP-3) mRNA and peptide expression in several cell types,

and TGF- $\beta$  induced growth inhibition and apoptosis have been shown to be mediated through the induction of IGFBP-3.

The aim of this study was to compare the effects of TGF- $\beta$  on cell growth inhibition and modulation of IGFBP-3 production in normal human mammary MCF-10A cells versus Hs578T human breast cancer cells.

MCF-10A and Hs578T cells were treated with 5 ng/ml TGF- $\beta$  for five days. Growth inhibition was assessed by trypan blue exclusion and endogenous IGFBP-3 monitored and measured by western immunoblotting and radioimmunoassay, respectively, of the conditioned media (CM). Dosing was repeated as above in the Hs578T cells with 20  $\mu$ g/ml antisense mRNA to IGFBP-3 added on days 1, 3, and 5 with the same assessments. In addition, MCF-10A cells were treated with exogenously added IGFBP-3 (100–200 ng/ml) for 24 hours and cell number assessed as before.

In Hs578T and MCF-10A cells, TGF- $\beta$  alone caused no cell death but a 53% and 62% inhibition of cell growth, respectively. In the Hs578T cells, cell growth inhibition was accompanied by a significant increase in endogenous IGFBP-3 from 77 ng/ml in control cells to 906 ng/ml ( $p < 0.001$ ). Coincubation with antisense mRNA to IGFBP-3 significantly decreased IGFBP-3 concentrations to 370 ng/ml ( $p < 0.001$ ) and abrogated the growth inhibitory effect of TGF- $\beta$ . In the normal mammary cells, TGF- $\beta$  similarly induced significant cell growth inhibition, but in contrast to the Hs578T cells this was accompanied by a dramatic decrease (97%) in endogenous IGFBP-3 values. Conversely, treatment of these cells with exogenously added IGFBP-3 caused a significant increase in cell proliferation (approximately 50%).

TGF- $\beta$  induces cell growth inhibition in both normal and cancerous mammary epithelial cells. However, this appears to be effected by increasing endogenous levels of IGFBP-3 in Hs578T cells but by decreasing IGFBP-3 levels in MCF-10A cells. These data suggest that IGFBP-3 has differential effects on cell growth, being able to promote growth in normal mammary epithelial cells but to inhibit the growth of breast cancer cells.

#### Abstract 37. Serum concentrations of IGF signalling peptides at diagnosis of acute lymphocytic leukaemia (ALL) predict relapse risk

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The long term prognosis of children with lymphoid neoplasia depends both on the relapse risk and on side effects of treatment. At the time of diagnosis only a few parameters—age, immunophenotype, prednisone response—have been of predictive value in identifying those patients who need intensified and those who need less toxic treatment. In this study, patients with high risk (HR-) ALL were excluded. The remaining 148 children with standard or medium risk ALL treated by the Berlin-Frankfurt-Münster (BFM) study group were analysed

for the predictive value of serum values of insulin-like growth factor I (IGF-I), IGF-II, and IGF binding proteins (IGFBPs) 1, 2, and 3 at diagnosis to determine the two year relapse free rate. At diagnosis of ALL we found raised IGFBP-2 (median/min to max: +3.2 SDS/−7.5 to 8.1), low IGF-I (median/min to max: −2.3/−9.3 to 0.7) and IGFBP-3 (median/min to max: −1.3/−12.3 to 2.5), but normal IGFBP-1 (median/min to max: +0.2/−2.4 to 4.4) and IGF-II (median/min to max: −1.0/−5.7 to 5.0). One hundred and thirty four patients were followed for at least two years in continuous complete remission and 14 developed a relapse. Using cluster analysis, serum concentrations of IGFBP-1, IGFBP-2, and IGF-II at diagnosis differentiated two groups with low relapse risk (1/69 v 13/79). Therefore, in addition to conventional risk factors, serum concentrations of IGFBP-2, IGFBP-1, and IGF-II at the time of diagnosis predict continuous complete remission in a subgroup of ALL. In addition, raised IGFBP-2, IGFBP-1, and low IGF-II at diagnosis indicate a higher relapse rate, implying resistance to chemotherapy. We hypothesise that low systemic IGF-I and IGF-II were compensated for at sites of clonal expansion by a local accumulation of IGFBP-2/IGF complexes.

#### Abstract 38. Genomic imprinting of IGF-2 and H19 in human meningiomas

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Several genes, such as the gene encoding insulin-like growth factor 2 (IGF-2) and H19, are normally imprinted with preferential expression of the paternal or maternal allele, respectively. Loss of imprinting (LOI) of IGF-2 and H19 is found in several tumours, suggesting that LOI of IGF-2 and/or H19 may play an important role in tumorigenesis. The IGF-2 gene encodes a fetal growth factor and the H19 gene is likely to act as an RNA with an antitumour effect. We investigated the imprinting status of IGF-2 and H19 in human meningiomas. The IGF-2 gene, which is normally imprinted, is not imprinted in the leptomeninges and choroid plexus of the brain. To examine the imprinting status of IGF-2 and H19 in human meningiomas we used the ApaI polymorphisms in exon 9 of the IGF-2 gene and the AluI polymorphism in exon 5 of the H19 gene. In total, 24 meningiomas of WHO grade I, II, and III were analysed. Fifteen meningiomas (63%) were informative for the ApaI polymorphism in the IGF-2 gene. Monoallelic expression (MAE) for IGF-2 was found in 11 of 15 tumours (73%), which is in contrast to the lack of imprinting status of IGF-2 in leptomeninges. Ten cases (42%) were heterozygous for the H19 gene and biallelic expression was found in three of 10 meningiomas (30%). The results indicate that modulation of the imprinting status of IGF-2 and H19 may play an important role for the development of meningiomas.

#### Abstract 39. The CCN gene family and cancer: structural or functional relation with IGFBPs?

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The prototypic members of the CCN family (CTGF, CYR61, and NOV) were discovered in the early 1990s. Additional members of the family have been identified, including Elm-1/WISP-1, WISP-3, and Cop-1/WISP-2. These highly conserved cysteine rich proteins share four modular domains (each encoded by a single exon) with sequence similarities to insulin-like growth factor binding protein (IGFBP), von Willebrand factor, thrombospondin, and a cysteine knot characteristic of some growth factors including platelet derived growth factor (PDGF), nerve growth factor (NGF), and transforming growth factor  $\beta$  (TGF- $\beta$ ). Cop-1/WISP-2 is unique because it lacks the C-terminal cysteine knot domain.

Purified CCN proteins have been shown to mediate and promote cell adhesion, migration, proliferation, and survival. Matrix associated, heparin binding proteins CTGF and CYR61 are novel ligands of integrins  $\alpha\beta 3$  and  $\alpha IIB\beta 3$ , and NOV interacts with fibulin 1C, suggesting their involvement in cell adhesion signalling. Both CTGF and CYR61 induce angiogenesis in vivo and chondrogenesis in vitro. CTGF is expressed in fibroblasts during wound healing and can induce fibrosis in vivo. Furthermore, CTGF has been demonstrated to mediate both the mitogenic and matrigenic activities of TGF- $\beta$ . Other studies have revealed that CYR61 promotes tumour growth, whereas Cop-1 or Elm-1/WISP-1 can inhibit tumour growth. It has also been established that the expression of NOV is abnormal in tumour cells and that the expression of an amino truncated form of NOV is transforming but full length NOV inhibits fibroblast growth, suggesting an involvement of this proto-oncogene in malignancy.

The expression and possible roles of CCN genes in cancer cells will be discussed with respect to the structural relation existing between IGFBPs and CCN proteins.

#### Abstract 40. IGF physiology and cancer risk: interpreting the data

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It is now approximately 30 months since the first prospective studies provided data suggesting that individuals with circulating insulin-like growth factor I (IGF-I) values at the high end of the normal range have a higher risk for common cancers than individuals at the lower end of the normal range. Earlier but smaller and non-prospective case control studies had reached similar conclusions. Most of the subsequent studies during the past two years have confirmed the trend, although the quantification of the risk has varied between studies. In some studies, the strength of the association is as strong as that described for the link between cholesterol and cardiovascular disease.

Further data are required to describe better the nature of the relation between various IGF related analytes in the circulation and cancer risk, and many groups are now

studying this issue, using sample sets from repositories around the world.

The physiological basis of the relation remains unclear. We have hypothesised that in view of the well known fact that there is substantial variation in IGF-I values among normal individuals, this biological variation actually influences the carcinogenic process in a subtle way over decades of life (perhaps survival of partially transformed cells is higher in "high IGF-I" individuals, for example), and this leads to the observed association. An alternative hypothesis proposes that the IGF-I-risk relation is observed because even early neoplasms can raise circulating IGF-I values. Workers in this field must clearly consider the differences between a "tumour marker" and a "cancer risk factor". In addition, careful attention must be given to the stage of cancer in population studies, because it is well known that cachexia from any cause is associated with low IGF-I values. This explains some discrepancies in the literature: while subjects destined to have a diagnosis of cancer in the future and/or those just diagnosed with early cancer tend to have higher IGF-I levels than controls, subjects with advanced cancer have lower IGF-I levels as a consequence of malnutrition, cachexia, and liver disease.

Apart from generating data to describe the IGF physiology-cancer risk association better, ongoing work in the field is expanding in several directions that may have relevance to cancer prevention, namely: (1) studies of interaction between IGF related risk and "classic" risk factors, (2) characterisation of genetic and non-genetic factors that underlie person-to-person variability in circulating analytes related to IGF physiology, and (3) better understanding of the physiological basis for the unexpected discordance between IGF-I and IGFBP-3 values consistently observed in a minority of apparently normal subjects.

**Abstract 41. The IGF-I/IGFBP-3 molar ratio predicts the presence of advanced colorectal adenomas in normal populations and possibly acromegalics**

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There is increasing evidence that physiologically high insulin-like growth factor I (IGF-I) and low IGF binding factor (IGFBP-3) levels within a population may predict the subsequent development of common epithelial cancers, including colorectal cancer. Most colorectal cancers arise from adenomas (the adenoma-carcinoma sequence), and different stages of development are recognised: early "low risk" and advanced "high risk" adenomas (size  $\geq$  1cm, tubulovillous or villous histology, severe dysplasia, or multiplicity). This report includes two parallel studies examining the relation of circulating IGF-I and IGFBP-3 with the different stages of adenoma development in two groups: a group of healthy adults and a group of acromegalics undergoing screening colonoscopy.

Study 1: within the randomised Flexi-Scope trial (healthy volunteers, aged 55 to 64 years), we measured serum IGF-I and

IGFBP-3 in sera collected prospectively from 442 attendants. Of these, 100 individuals underwent a complete screening colonoscopy: 47 normal examinations, 11 low risk adenomas, 42 high risk adenomas. Estimates of relative risk for the adenomatous stages were calculated using multiple logistic regression, adjusting for known risk factors.

In study II we also measured serum IGF-I and IGFBP-3 in 60 acromegalic patients (aged 24 to 80 years) at the time of screening colonoscopy. Because ages ranged in this group over six decades, age-sex adjustments were made using normative data from 295 healthy individuals. IGF-I/IGFBP-3 molar ratios were calculated for both studies.

Study I: mean serum IGF-I and IGFBP-3 values were similar in individuals with a normal colonoscopy and low risk adenomas. By contrast, serum IGF-I was increased (mean (SD), 190 (53) v 169 (54)  $\mu$ g/litre;  $p = 0.06$ ), and serum IGFBP-3 was significantly decreased (mean (SD), 3.22 (0.60) v 3.47 (0.62) mg/litre;  $p = 0.05$ ) in individuals with high risk adenomas compared with normal colonoscopy and low risk adenomas combined. The resulting IGF-I/IGFBP-3 molar ratio was significantly raised in individuals with high risk adenomas (mean (SD), 0.26 (0.06) v 0.22 (0.08);  $p = 0.002$ ). There were strong correlations between repeated samples for both IGF-I ( $r = 0.74$ ;  $p < 0.001$ ) and IGFBP-3 ( $r = 0.82$ ;  $p < 0.001$ ), and values were unaffected by the removal of adenomas. With high risk adenoma as the dependent factor, regression models demonstrated a significant positive association with IGF-I after controlling for IGFBP-3 (relative risk (RR)/1 SD change = 4.39; 95% confidence interval (CI), 1.31 to 14.7;  $p = 0.02$ ), and independently, an inverse association with IGFBP-3 after adjustment for IGF-I (RR/1 SD change = 0.41; 95% CI, 0.20 to 0.82;  $p = 0.01$ ).

Study II: by simple comparisons between acromegalics with ( $n = 6$ ) and without advanced adenomas, there were no differences in IGF-I/IGFBP-3 molar ratios. However, when plotted on age-sex adjusted curves, trends emerged, although the numbers were too small to be conclusive.

The findings from these parallel studies suggest that the IGF-I/IGFBP-3 molar ratio predicts future colorectal cancer risk in the normal population and possibly in acromegalic patients. Specifically, circulating IGF-I and IGFBP-3 may predict adenoma progression. These data support recent observations from the nurses' health study demonstrating similar associations (Giovannucci *et al. Cancer Epidemiol Biomarkers Prev* 2000;9:345-9)—the current study is smaller but its strengths include the setting within a randomised trial, a well defined age range, and adenoma classification using uniform histological criteria.

**Abstract 42. IGF-II peptide overexpression early in the adenoma-carcinoma sequence of colorectal neoplasia in humans and the Min mouse model**

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We have reported previously that serum insulin-like growth factor II (IGF-II) values

are raised in individuals with colorectal adenomas (Renehan AG, *et al. Clin Endocrinol Metab* 2000 [In press.]), and in patients with early stage colorectal cancer (Renehan AG, *et al. Br J Cancer* 2000;83:1344-50). Other studies have shown that IGF-II mRNA is minimally expressed in normal adult colonic epithelium, but is overexpressed in some human colonic adenocarcinomas and colonic cancer cell lines. We tested the hypothesis that IGF-II peptide overexpression may occur early in the pathogenesis of colorectal neoplasia using the adenoma-carcinoma model in humans and the Min (multiple intestinal neoplasia) mouse model. In addition, as IGF-II is potentially mitogenic and anti-apoptotic via the IGF-I receptor, we investigated the association between IGF-II expression and cell proliferation and apoptotic indices in the neoplastic tissues.

The human material consisted of the following: (1) well orientated small intestinal epithelium obtained from individuals without colonic neoplasia ( $n = 10$ ), (2) well orientated colonic epithelium obtained from individuals without colonic neoplasia ( $n = 10$ ), (3) "normal" colonic epithelium from patients undergoing colonic resection for cancer ( $n = 12$ ), (4) hyperplastic polyps ( $n = 10$ ), (5) adenomas ( $n = 66$ ), and (6) adenocarcinomas ( $n = 70$ ). We also included well orientated colonic epithelium ( $n = 10$ ) and adenomas ( $n = 18$ ) from acromegalic patients. The mouse study comprised intestinal bundles ( $\times 2$ ) at 8, 14, 16, 24, and 29 weeks after birth. Immunohistochemical staining was performed using antibodies to IGF-II. Tumour expression was scored semiquantitatively, as follows: 0, negative; 1,  $< 25\%$ ; 2, 26-75%; 3,  $> 75\%$ , and normal epithelium scored using positional cell analysis. Peptide overexpression was quantified using western immunoblots and radioimmunoassays of homogenates. Cell proliferation and apoptotic indices were calculated following MIB-1 and M30 cytodeath staining, respectively.

There was no IGF-II immunorexpression in the small intestine, normal colonic epithelium from non-neoplastic patients (sporadic and acromegalic), or hyperplastic polyps. In contrast, IGF-II expression was observed in "normal" basal crypt epithelium from cancer patients corresponding with maximum anti-apoptotic activity, and was expressed intensively in dysplastic crypts adjacent to adenocarcinomas (31 of 38 informative cases). IGF-II positivity was observed in 83% of all adenomas; notably, IGF-II positivity was recorded in 80% of adenomas  $< 5$  mm in size. For all adenocarcinomas, IGF-II positivity was 53%, rising to 85% for well differentiated adenocarcinomas. Serial examination of the Min mice intestines demonstrated that IGF-II was expressed in microcystic adenomatous lesions (the earliest lesions), but that it was absent in normal intestinal epithelium.

For human neoplastic lesions, IGF-II scoring showed no association with cell proliferation, but demonstrated an inverse association with apoptotic rates in adenomas (table 2).

This study has confirmed that IGF-II peptide expression is absent or minimal in normal colonic epithelium but, by contrast, has shown that IGF-II is overexpressed early in the adenoma-carcinoma sequence of colorectal cancer in humans and the Min mouse model. These findings support the hypothesis

Table 2 Abstract 42. Relation between IGF-II scores and cell proliferation and apoptosis

| IGF-II expression | Adenoma    |               | Adenocarcinoma |               |
|-------------------|------------|---------------|----------------|---------------|
|                   | Apoptosis  | Proliferation | Apoptosis      | Proliferation |
| Scores 0/1        | 3.6 (0.8)  | 18.7 (3.5)    | 9.3 (1.0)      | 54.3 (5.4)    |
| Scores 2/3        | 1.4 (0.8)* | 20.4 (3.6)    | 8.1 (0.9)      | 44.6 (5.0)    |

Values are mean (SE).

\*p, 0.001.

IGF-II, insulin-like growth factor II.

that raised IGF-II in the circulation may reflect direct adenoma expression, and thus behave as a potential tumour marker. In addition, the anti-apoptotic properties rather than the stimulatory effects of IGF-II may be more relevant in colorectal carcinogenesis.

#### Abstract 43. Investigation of the dependency of renal cancer cells on IGFs

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Kidney cancer is a malignant disease associated with a poor prognosis. In the USA, the incidence is in the order of 30 000 new cases annually. It is well known that several cancer forms are associated with insulin-like growth factor (IGF) secretion from tumour cells, which is used for autocrine growth stimulation. It is also known that blocking the IGF receptor may lead to apoptosis.

The present study has investigated the biological role of IGF-I in malignant epithelial kidney carcinoma cells in vitro. The cell lines investigated were Caki-2 and Gurall and the effects were compared with previously described cell lines, such as the breast cancer cell line MCF-7. The cells showed a strong proliferative response to IGF-I, IGF-I analogues, and IGF-II, but not to the otherwise functionally related epidermal growth factor (EGF), indicating that these cells are dependent on IGF to progress through the cell cycle. In experiments where neutralising antibodies to the IGF type I receptor were added to the culture medium, the cells showed a greatly reduced capacity to initiate proliferation. Interestingly, the addition of soluble IGF-I receptor to the culture medium resulted in strong inhibition of the proliferative response in Gurall cells, but had no marked effect on Caki-2 cells, indicating different cellular regulation in these two epithelial cell lines. The effects of these different ways of blocking the receptor had different impacts on both IGF binding protein (IGFBP) secretion and IGF-I dependent rescue from apoptosis.

Our present results show that the IGF family plays an important role in the progression of these malignant cells. Thus, interference in this system may be a novel approach in kidney cancer treatment.

#### Abstract 44. Expression of IGF-2 and IGFBPs 2, 5, and 6 in meningiomas with different brain invasiveness

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Meningiomas show clinical characteristics that vary from very benign to clearly malignant with rapid invasive growth and metasta-

sis. Fast growing meningiomas appear to have increased numbers of Ki-67 positive cells, but other histochemical markers of aggressive clinical behaviour are largely unknown. This study was undertaken to analyse the gene expression of members of the insulin-like growth factor (IGF) family in meningiomas showing different degrees of brain invasion. We have previously found a strong correlation between anaplastic/atypical histopathology and a high IGF-2/IGFBP-2 mRNA ratio in sporadic meningiomas. A strong correlation was also found between clinical outcome and IGF-2/IGF binding protein 2 (IGFBP-2) ratio.

In this study, meningiomas were selected depending on their pattern of brain invasion and brain oedema. The tumours comprised three groups: (1) Benign meningiomas that did not interfere with the arachnoid plane and showed no oedema. (2) Benign meningiomas that invaded the arachnoid plane and reached the pia mater. These tumours caused oedema. (3) Aggressive and malignant meningiomas that caused oedema and showed brain invasion. Tissue samples were analysed by in situ hybridisation.

The expression of IGF-2 was high in all tumours analysed, but a clear increase in expression was observed in group 3, with anaplastic or aggressive meningiomas. IGFBP-2 expression was, to our surprise, found to be rather low in all tumours, and there was no correlation with tumour behaviour. IGFBP-5 and IGFBP-6 mRNA levels were strongly correlated with tumour behaviour. IGFBP-6 mRNA was highest in the group with brain invasion; these were patients with anaplastic or aggressive meningiomas. Patients with arachnoid invasion and ensuing oedema had lower IGFBP-6 concentrations. Patients with intact arachnoid membranes and no oedema had very low concentrations of IGFBP-6. On the other hand, IGFBP-5 mRNA was highly expressed in the more benign group of meningiomas and low mRNA levels of IGFBP-5 were detected in the group with brain invasion. These two molecules thus constitute additional molecular markers for tumour progression.

How these two molecules are regulated, and how/if they regulate the function of IGF-2 need to be further investigated. However, IGFBP-5 has been shown to associate with the extracellular matrix and to be degraded by matrix metalloproteinases (MMPs). In this selection of meningiomas we have found a correlation between MMP-9 expression and brain invasion. Higher mRNA levels of MMP-9 were found in group 3 (patients with aggressive meningiomas causing oedema and showing brain invasion) compared with group 1 and 2 meningiomas. This suggests that IGFBP-5 is regulating the levels of IGF-2 mRNA in more benign meningiomas, whereas MMP-9 may degrade and/or downregulate IGFBP-5 in anaplastic or aggressive meningiomas.

IGFBP-6 has a high affinity for IGF-2 and is here suggested to attract IGF-2 to the tumour cells, which may result in stimulation of new synthesis of IGF-2 in an autocrine fashion. The overexpression of IGF-2 would thereby increase the risk of poor patient prognosis.

#### Abstract 45. A new autocrine loop involving IGF-II and the isoform A of the insulin receptor in thyroid cancer

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Thyroid cancer originating from the follicular epithelium is represented by well differentiated tumours (papillary or follicular) in over 90% of cases and by undifferentiated tumours in less than 10% of cases. Cancer related mortality ranges from approximately 10% in differentiated tumours up to 100% in undifferentiated tumours. The mechanisms determining thyroid cancer aggressiveness are incompletely understood and may include local production of peptide growth factors and overexpression of cognate tyrosine kinase receptors.

We recently observed that one of the two insulin receptor (IR) isoforms (IR-A isoform) is predominantly expressed in fetal tissues and malignant cells and binds IGF-II with high affinity. Because IRs are overexpressed in most thyroid tumours, in the present study we explored the possibility that the overexpressed IRs could be activated by locally produced insulin-like growth factor II (IGF-II) and could contribute to thyroid cancer progression.

We measured IGF-II production, IR content, and the relative abundance of the two isoforms both in normal (n = 11) and cancer cell (n = 8) primary cultures.

IGF-II was expressed in all cancer cells and highly expressed in anaplastic cells but present only in trace amounts in normal cells. IRs were overexpressed in thyroid cancer cell cultures as compared with normal thyroid cell cultures (4.3–52.6 v 1.2–1.7 ng/100 µg protein, respectively). In addition, IRs were predominantly present as the IR-A isoform in cancer cells and as IR-B in normal thyroid cells. The relative IR-A abundance ranged from 36% to 70% in cancer cells (with the highest values in undifferentiated cancers) compared with 27% to 39% in normal cells. Exogenous IGF-II caused IR autophosphorylation with an ED<sub>50</sub> of 1.5–40.0 nM in cancer cells v > 100 nM in normal cells; IGF-II affinity correlated with the relative abundance of IR-A (r = 0.628, p < 0.0001).

In conclusion, we identified in thyroid cancer a novel autocrine loop involving malignant cell IGF-II production and IR-A overexpression. This loop, absent in normal thyroid cells, greatly increases with cell differentiation and may, therefore, affect thyroid cancer progression and aggressiveness.

**Abstract 46. Analysis of the IGF axis in preneoplastic hepatic foci and hepatocellular neoplasms developing after low number pancreatic islet transplantation into the livers of streptozotocin diabetic rats**

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Preneoplastic hepatic foci have been demonstrated in liver acini draining the blood from intraportally transplanted pancreatic islets in streptozotocin induced diabetic rats with mild persisting diabetes. In long term studies of this animal model, hepatocellular adenomas and carcinomas (HCCs) developed after a sequence of characteristic preneoplastic hepatic foci. In this experimental model, the local hyperinsulinism has been suggested to have a causative role. Because of the close link between insulin and the insulin-like growth factor (IGF) axis altered gene expression of an IGF axis component is likely to occur. Therefore, preneoplastic hepatic foci as well as HCCs were studied for the expression of IGF axis components. Glycogen storing, "early" preneoplastic hepatic foci were already detectable several days after pancreatic islet transplantation. Northern blot analysis, *in situ* hybridisation, and immunohistochemical studies of these early lesions demonstrated an increased expression of IGF-I and IGF binding protein 4 (IGFBP-4) in altered parenchymal cells, whereas that of IGFBP-1 was greatly downregulated. IGF-II was not detectable in these preneoplastic foci. HCCs arising in this model exhibited a decreased expression of IGF-I and IGFBP-4, whereas IGFBP-1 was not significantly altered. Some HCCs showed a more than 100-fold overexpression of IGF-II whereas other tumours were completely negative. A low IGF-I receptor expression was detected in preneoplastic foci and adjacent non-altered liver tissue. However, HCC tissue consistently showed increased IGF-I receptor expression rendering these tissues susceptible to the mitogenic effects of IGFs. The altered gene expression in glycogen storing preneoplastic hepatic foci, especially the upregulation of IGF-I and IGFBP-4 together with a downregulation of IGFBP-1, resemble the insulin dependent regulation of these components in normal rat hepatocytes. These data are in line with previous studies demonstrating a correspondence of the focal character, morphology, and enzymic pattern of preneoplastic hepatic foci with the insulin effects on hepatocytes. The step-wise development from preneoplastic foci to HCCs may be driven by insulin itself and/or altered IGF axis components, or yet unidentified factors.

**Abstract 47. Multiple genetic and epigenetic lesions in genes at 11p15.5 and the role of IGF2 dysregulation in Beckwith-Wiedemann syndrome**

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Beckwith-Wiedemann syndrome (BWS) is a complex fetal overgrowth syndrome resulting in a discrete set of developmental abnormalities of which large pre/postnatal size (> 90th centile), anterior abdominal wall defect, and macroglossia are the major manifestations. These are frequently associated with a variety of other anomalies including organomegaly, perinatal hypoglycaemia, hemihypertrophy, and a predisposition to developmental tumours, particularly Wilms' tumour.

The syndrome occurs in both sporadic and familial forms, both of which implicate a cluster of imprinted genes on the distal part of chromosome 11, 11p15.5. This cluster includes the maternally expressed CDKN1C cyclin dependent kinase inhibitor, KCNQ1, a voltage gated potassium channel, H19, IMPT-1, IPL, ASCL-2, and CD81 (in the mouse), and the paternally expressed IGF2, MTR1, and KCNQ10T genes. Several non-imprinted genes lie within this cluster, notably INS, which in humans has not been shown to exhibit allele specific expression. The aetiology of BWS is complex. Around 20% of sporadic cases show paternal uniparental disomy for this region of chromosome 11, resulting in biallelic expression of IGF2 and silencing of other normally maternally expressed loci; effectively, conversion to the paternal epigenotype. Several cases show IGF2 loss of imprinting (LOI), in the absence of disomy, associated with extinction of H19 expression and abnormal H19 promoter methylation, indicating the presence of an imprinting control centre (BWSIC1) controlling IGF2 and H19 imprinting at the telomeric end of this gene cluster. However, cases have also been found where IGF2 LOI is associated with normal H19 expression. This suggests that IGF2 is further subject to control from another region: BWSIC2.

Investigation of breakpoint cluster regions centromeric to IGF2 (BWSCR2) implicated the very large gene KCNQ1 in the aetiology of the syndrome. An antisense transcript associated with a differentially methylated CpG island within an intron of the gene was found to be defective in a large number of sporadic patients with loss of methylation (LOM) at this position and loss of imprinting of the antisense transcript. In some cases this LOM at KCNQ10T is associated with LOI of IGF2, suggesting co-regulation of imprinting by a second imprinting centre: BWSIC2. Phenotypic evidence suggests that lesions at BWSIC2 may also be associated with LOI at the centromere proximal CDKN1C gene.

Previous studies have suggested that BWS patients with uniparental disomy are at increased risk of developing developmental tumours. It is interesting therefore that although LOI at IGF2, an expected consequence of BWSIC1 lesions, is frequently associated with Wilms' tumours of both syndromic and sporadic origins, LOI or mutation of KCNQ10T, consistent with a BWSIC2 lesion, is not seen, despite it being

associated with IGF2 LOI in patients with BWS. This apparent contradiction clearly requires further study into the specific details of the molecular lesions in BWS subgroups, but the conclusion that overexpression of IGF2 is sufficient to cause these developmental tumours is clearly oversimplified. Epigenetic extinction of CDKN1C expression might also be expected to contribute to tumour formation, particularly because there is evidence of convergence of the growth pathways controlled by IGF2 and p57<sup>KIP2</sup>. However lesions in CDKN1C are rare in Wilms' tumours. It is not known whether IGF2 LOI actually raises concentrations of stable IGF-II protein in patients with BWS because of a lack of appropriate fetal material. We have undertaken an immunohistochemical study of IGF2 levels in a small series of late fetal and perinatal tissues from individuals with BWS. We demonstrate that although concentrations of IGF-II do not differ dramatically from normal controls, the expression of IGF2 is strikingly maintained in all the tissues examined for much longer in the later fetal period than in controls, suggesting that a crucial factor in the outcome of IGF2 LOI is not the concentration of peptide achieved but dysregulation of the timing of expression.

**Abstract 48. The IGF-I receptor: an effective therapeutic target in Ewing's sarcoma**

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Ewing's sarcoma (ES) ranks second in frequency among primary bone tumours. It is an extremely aggressive, poorly differentiated neoplasm of uncertain histogenesis, usually arising in children and young adults. Despite the use of multimodal treatments and of very aggressive chemotherapeutic regimens, the long term disease free survival of patients with ES is still disappointingly low, particularly in high risk groups. The identification of valuable, new therapeutic targets for the design of innovative, more effective strategies is therefore urgently needed for this tumour. In recent years, by analysing the role of growth factors in the pathogenesis of ES, we have reported the autocrine production of insulin-like growth factor I (IGF-I) and the constant presence of its corresponding receptor (IGF-IR) in ES cells. The blockage of the IGF-IR mediated circuit by  $\alpha$ IR3 monoclonal antibody (MAb), which specifically neutralises IGF-IR, greatly inhibits the growth and the migration ability of ES cells *in vitro*, as well as their tumorigenic and metastatic ability *in vivo*. A significant inhibition of ES growth has also been successfully achieved *in vivo* by using suramin, a non-specific growth factor antagonist that inhibits several autocrine circuits, including the IGF-IR mediated loop. Therefore, impairment of IGF-IR appears to be a valuable therapeutic approach against ES. However, from a clinical point of view, to be of practical value targeted therapy should be effectively combined with chemotherapeutic drugs. Because active IGF-IR is emerging as a powerful inhibitor of apoptosis induced by a variety of agents, we have tested the effectiveness of strategies aimed at IGF-IR impairment to enhance the sensitivity to anticancer agents. We stably transfected antisense IGF-IR

sequences or dominant negative IGF-IR mutants into the human ES cell line TC-71. Antisense transfectants as well as clones expressing dominant negative IGF-IR mutants showed a significantly reduced survival and growth in anchorage independent conditions, further supporting the role of IGF-IR in ES malignancy. In addition, chemosensitivity against doxorubicin and vincristin, two leader drugs in the treatment of ES patients, was significantly enhanced in transfectants as well as in cells treated with  $\alpha$ IR3 MAb. Taken together, these findings indicate that the inhibition of IGF-IR by blocking antibodies, antisense strategies, or dominant negative mutants of IGF-IR is a promising strategy to be combined with conventional cytotoxic drugs for the design of more effective therapeutic regimens in patients with ES.

#### **Abstract 49. Expression of IGF-1, IGF-1R, and IGFBP-3 in benign and malignant thyroid tissue**

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Normal cells demonstrate a strictly limited growth potential and senescence after a defined number of cell divisions. In contrast, tumour cells escape from the mechanism controlling the balance between proliferation and cell death, and therefore often exhibit an apparently unlimited proliferation potential. Insulin-like growth factor 1 (IGF-1) is a major proliferation and survival factor and the expression of IGF-1 and the IGF-1 receptor (IGF-1R) is implicated in the malignant growth of many cancers. Their proliferative activity is influenced by their binding proteins (IGFBPs). Thyroid cancer is the most frequent malignancy of the endocrine system. Thyrocytes can develop in three different forms of malignant cancer with different growth and metastasis behaviour. The aim of the present investigation was to study whether the expression of IGF1, IGF-1R, and IGFBP-3 could serve as a marker of dedifferentiation in human thyroid carcinomas.

Total RNA was isolated from 10 papillary, 10 follicular, and 10 undifferentiated thyroid carcinomas as well as 10 goitre tissues. For reverse transcription polymerase chain reaction (RT-PCR) analysis specific primers were used (IGF-1: sense 5'-ATC CTT CTC TCC TCA TTC TTC-3', antisense 5'-GAT ACA CAG ACA CAG ATA AAA G-3'; IGF-1R: sense 5'-TCC ACA TCC TGC TCA TCT CC-3', antisense 5'-AGA AGT CAC GGT CCA CAC AG-3'; and IGFBP-3: sense 5'-TCA GAG CAC AGA TAC CCA G-3', antisense 5'-ACA GCC GCC TAA GTC AC-3'). In addition, frozen sections of all tissues were prepared for immunohistochemical staining using commercially available polyclonal antibodies.

RT-PCR analysis revealed that transcripts of IGF-1, IGF-1R, and IGFBP-3 were found in 90%, 83%, and 90% of thyroid cancers, respectively. There was no difference between the papillary, follicular, and undifferentiated thyroid carcinomas. IGF-1 and IGF-1R mRNA was detected in all of the goitre tissues. The expression of IGFBP-3 transcripts was very low in goitre tissue when compared with cancer tissues. IGF-1 immu-

noreactivity was seen in 70% and IGF-1R in 83% of tumour tissue, whereas the corresponding values were 80% and 90% in the goitre tissue. However, IGFBP-3 immunoreactivity was significantly higher in tumours when compared with goitre tissue (77% *v* 30%). IGF-1 protein was located in stromal cells as well as in thyrocytes. Our data show that the transcript is well correlated with the protein expression. IGF-1 and IGF-1R were detected in most thyroid tissues but IGFBP-3 was strongly expressed in thyroid carcinomas.

The high IGFBP-3 expression in cancer tissue might be indicative of a role for this protein in regulating the growth of thyroid carcinoma.

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#### **Abstract 50. Evolution of IGF-IR signalling during breast cancer progression**

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The insulin-like growth factor I receptor (IGF-1R) is a ubiquitous transmembrane tyrosine kinase that may control different growth related and unrelated processes, such as proliferation, survival, neoplastic transformation, migration, and adhesion.

Recent clinical and experimental data implicate IGF-1R in the development of breast cancer. IGF-1R is up to 14-fold overexpressed in oestrogen receptor (ER) positive breast cancer cells compared with normal epithelial cells. IGF-1R ligands are strong mitogens for many breast cancer cells in vitro, and higher concentrations of circulating IGF-I correlate with breast cancer risk in premenopausal women. In addition, increased expression of either IGF-1R or its major signalling substrate IRS-1 have been linked with increased drug and radioresistance and cancer recurrence at the primary site. In vitro experiments demonstrated that the IGF-1R/IRS-1/PI-3K pathway provides strong growth and survival responses in ER positive cells.

The functions and signalling of IGF-1R in ER negative breast cancer cells are still obscure. ER negative tumours and cell lines express low levels of IGF-1R, and do not respond to IGF-I with growth. However, despite the lack of an IGF-I mitogenic response, the metastatic potential of ER negative breast cancer cells can be effectively inhibited by different compounds targeting IGF-1R. This suggests that IGF-1R must control some events that are crucial for metastatic cell spread.

Consequently, we investigated the possibility that in ER negative metastatic breast cancer cells IGF-1R selectively promotes growth unrelated processes, such as migration and invasion, but is not engaged in the transmission of growth and survival signals. Using ER negative MDA-MB-231 breast cancer cells and their IGF-1R overexpressing derivatives, we demonstrated that IGF-I acts as a chemoattractant for these cells. The extent of IGF-I dependent migration reflected IGF-1R concentrations and required the activation of phosphatidylinositol 3-kinase (PI-3K) and p38 (but not ERK1/ERK2) kinases. The same pathways promoted IGF-I dependent motility in ER positive MCF-7 cells.

In contrast to the positive effects on cell migration, IGF-I was totally unable to improve the growth and survival of MDA-MB-231 cells, while it was a potent mitogen for MCF-7 cells. Investigating the molecular basis of this phenomenon, we found that the impaired IGF-I mitogenicity in ER negative cells was not caused by the low IGF-1R expression, defective IGF-1R tyrosine kinase activity, or improper tyrosine phosphorylation of IRS-1. Also, the acute (15 minutes) IGF-I stimulation of PI-3 and Akt kinases was similar in ER negative and ER positive cells. Interestingly, the long term (two days) activation of the Akt pathway occurred only in MCF-7 cells, whereas it was significantly impaired in MDA-MB-231 cells. However, reactivation of this pathway by overexpression of constitutively active Akt mutants was not sufficient to improve growth or survival (with or without IGF-I) in ER negative cells, suggesting that other (unknown) pathways are also required for the mitogenic IGF-I response.

In summary, our results suggest that IGF-1R function undergoes evolution during breast cancer progression from the ER positive to the ER negative phenotype: growth related signalling becomes attenuated, whereas non-mitogenic processes such as migration are still under IGF-1R control.

#### **Abstract 51. The importance of the IGF-I system for cell proliferation, apoptosis, and chemoresistance**

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The insulin-like growth factor (IGF) system encompasses several proteins that form a highly regulated network of interactions. A simplified representation of the IGF system lists three receptors (IGF-1R, IGF-2R, and the insulin receptor), three ligands (IGF-I, IGF-II, and insulin), at least six IGF binding proteins (IGFBPs), and various binding protein proteases.

IGF-I exerts pleiotropic effects on mammalian cells via stimulation of its receptor, a receptor tyrosine kinase. In vivo, IGF-I acts both as a local tissue growth factor and as a circulating hormone. In oncological research, IGF-I has received increased attention because the activated IGF-I/IGF-1R system displays proliferative and anti-apoptotic properties in various cell types by stimulating distinct intracellular signalling pathways. Recent case control studies have found an approximate 10% increase in IGF-I serum concentrations in patients with lung, prostate, and breast cancer. These studies indicate an association between serum values and cancer risk. However, causality has not been established yet. Recent data, including our own on testicular carcinoma cell lines, suggest that the anti-apoptotic effect of IGF-I may mediate resistance to chemotherapeutic drugs in vitro and in vivo. Additional evidence for a role of the IGF-I system in the apoptotic network comes from the observation that the tumour suppressor gene p53 regulates the expression of the IGF-1R. In our cell line system, we observed an upregulation of p53 and downregulation of IGF-1R ( $\beta$ -chain) subsequent to the exposure to cytotoxic concentrations of cisplatin, which is accompanied by a significant induction of

apoptosis. Notably, besides its regulating properties on the IGF-I receptor, p53 possesses a direct binding site in the IGFBP-3 promoter. Thus, IGFBP-3 could contribute to the p53 dependent apoptosis in response to DNA damage. In summary, because of its possible role as a cancer risk factor and its functional properties in regulating the apoptotic machinery, targeting the IGF-I/IGF-IR system could serve as an approach in cancer prevention and to overcome clinical drug resistance in certain tumours.

#### Abstract 52. IGF signalling system in acute lymphoblastic leukaemia: new aspects in gene expression

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The role of insulin-like growth factors (IGFs), their specific binding proteins (IGFBPs), and binding protein related proteins (IGFBP-rPs) has been shown to be an integral part of the growth promotion in several neoplasms. Based on our previous reports about raised IGFBP-2 serum values in children suffering from acute lymphoblastic leukaemia (ALL) we studied the gene expression of members of the IGF signalling system in malignant lymphoblasts of these patients.

Bone marrow samples from more than 130 patients with ALL were investigated by reverse transcription polymerase chain reaction (RT-PCR). We found that at diagnosis IGFBP-2 was expressed in the malignant lymphoblasts of more than 60% of all ALL patients. In contrast to these findings, we found that only 30% of mononuclear cells were positive for IGFBP-2 expression in controls. We also demonstrated a clear correlation between IGFBP-2 expression in leukaemic cells and relapse in our patients. Eighty nine per cent of patients with a haematological relapse expressed IGFBP-2 in their lymphoblasts at diagnosis. Therefore, we conclude that there is a direct link between raised serum IGFBP-2 values in ALL patients, the expression of IGFBP-2 mRNA in the malignant lymphoblasts, and the relapse risk in these patients.

Furthermore, significant correlations of immunological subtype and the expression of components of the IGF signalling system were observed. Eighty five per cent of all lymphoblasts with a T cell origin were found to be IGFBP-2 positive, whereas 95% of all lymphoblasts with a T cell origin were negative for CTGF/IGFBP-rP2 expression.

#### Abstract 53. Determinants of circulating IGF-I and IGFBPs 1-3 in premenopausal women: physical activity and anthropometry

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Experimental studies in animals and cell lines have shown that insulin-like growth factor I

(IGF-I) is a potent mitogenic and anti-apoptotic agent, and IGF binding protein 3 (IGFBP-3) can increase apoptosis independent of IGF-I. In the circulation about 95% of IGF-I is bound to IGFBP-3 and a small proportion to IGFBP-1 and IGFBP-2. Epidemiological studies suggest that subjects with high serum concentrations of IGF-I and low concentrations of IGFBP-3 have a higher risk of colorectal cancer, prostate cancer, and premenopausal breast cancer. Endocrine IGF-I and IGFBP-3 values are not only influenced by growth hormone and age, but also by lifestyle factors and biological risk factors. The aim of our study was to assess whether physical activity and anthropometry are determinants of the endocrine IGF axis in a population of premenopausal women of similar age.

We conducted a cross sectional study in a population of 225 premenopausal women from the Prospect-EPIC study in the Netherlands. Highly active and highly inactive women were oversampled (n = 50 and n = 48, respectively). Concentrations of IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 were determined in non-fasting baseline plasma samples using immunoradiometric assays. On the day of baseline blood collection (1995-1997) height, weight, and waist and hip circumference were measured and information on habitual physical activity was assessed using a self administered questionnaire.

Mean concentrations of plasma IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 were 156.1, 14.3, 434.4, and 3062 ng/ml, respectively. The concentration of IGF-I correlated with the concentration of IGFBP-3 ( $r = 0.58$ ;  $p < 0.001$ ) and inversely correlated with concentrations of IGFBP-1 and IGFBP-2 ( $r = -0.26$  and  $r = -0.29$ , respectively;  $p < 0.001$ ). We observed lower plasma concentrations of IGF-I and IGFBP-3 in women in the low tertile of weight compared with those in the upper tertile (IGF-I:  $145 \pm 50$  v  $161 \pm 55$ ,  $p = 0.07$ ; IGFBP-3:  $2942 \pm 450$  v  $3134 \pm 463$ ,  $p = 0.01$ ). In contrast, concentrations of IGFBP-1 and IGFBP-2 were highest in women in the low tertile of weight. Similar results were found when women were grouped according to tertiles of body mass index, waist circumference, and waist to hip ratio. Women with a high physical activity score, composed of household work, sports, and other leisure time activities, also had somewhat lower IGF-I and IGFBP-3 concentrations and significantly higher IGFBP-1 and IGFBP-2 concentrations than women with a low physical activity score. Similar trends were observed with hours each week spent on sports activities between ages 20 and 40. If time permits, preliminary results will be presented for molar ratios of IGF-I and IGFBP-3, and for C-peptide and other diet related factors.

Our data suggest that a lean body shape and active lifestyle are associated with lower plasma concentrations of IGF-I and IGFBP-3 and with higher concentrations of IGFBP-1 and IGFBP-2. Bias in the assessment of physical activity by our questionnaire may explain why results are somewhat less consistent for physical activity. If future studies support a causal association between circulating concentrations of IGF-I (and binding proteins) and cancer risk, determinants of the endocrine IGF axis such as body size/shape, physical activity, and possibly dietary factors may provide new approaches for prevention strategies for several types of cancer.

#### Abstract 54. Structure of the insulin and IGF-1 receptors

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The insulin and epidermal growth factor (EGF) receptor families share considerable sequence identity. Their ectodomains have a similar arrangement of two homologous domains (L1 and L2) separated by a Cys rich region. The C-terminal half of the insulin receptor (IR) ectodomain is comprised of three fibronectin type 3 repeats, and an insert domain that contains the  $\alpha$ - $\beta$  cleavage site. The C-terminal portion of the epidermal growth factor receptor (EGFR) ectodomain consists solely of a second Cys rich region. The insulin and EGF receptors from some primitive multicellular organisms possess additional domains in both their extracellular and cytoplasmic regions.

We are engaged in an investigation of the structure of members of the insulin receptor family. We have shown that there is a single disulphide linking the  $\alpha$  and  $\beta$  chains of human IR and at least two  $\alpha$ - $\alpha$  disulphides involved in dimer formation. Single molecule electron microscope imaging of IR and IR-Fab complexes has revealed that the IR dimer resembles a compact U-shaped prism ( $90 \times 110 \times 120 \text{ \AA}$ ), where the L1-cysteine rich-L2 domains occupy the upper (membrane distal) region and the six fibronectin type 3 and two insert domains are located predominantly in the membrane proximal third.

Crystallisation of members of the IR family is hampered by the high level of glycosylation and attempts to obtain structural data from whole ectodomain have been unsuccessful. A fragment (residues 1-462) comprising the L1-cysteine-rich-L2 region of the IGF-1R ectodomain has been crystallised<sup>2</sup> and its structure determined to 2.6 Å resolution. The L domains each adopt a compact shape consisting of a single stranded right handed  $\beta$ -helix. The cysteine rich region is composed of eight disulphide bonded modules, seven of which form a rod shaped domain with modules associated in a novel manner. At the centre of this extended structure is a space, bounded by all three domains, and of sufficient size to accommodate a ligand molecule.

#### Abstract 55. IGF system and adrenocortical cancer

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The insulin-like growth factor (IGF) system is involved in the regulation of growth and differentiation of the adrenal gland. IGF ligands, receptors, and binding proteins are synthesised by adrenal glands of various species, and both IGFs induce steroidogenesis and mitogenesis in adrenocortical cells in vitro. Furthermore, there is substantial evidence that the IGF system is involved in adrenal tumorigenesis.

Overexpression of IGF-II has been found in human adrenocortical carcinomas (Gicquel *et al.* *J Clin Endocrinol Metab* 1994;78:1444), and recently we have demonstrated an overexpression of the intact IGF-I

receptor in three out of four adrenocortical cancers while the abundance of the IGF-I receptor in benign adrenal tumours was similar to normal tissue (Weber *et al. Eur J Endocrinol* 1997;136:296). However, the mechanism and functional relevance of the overexpression of IGF-II, and the IGF-I receptor in adrenocortical carcinomas remains unknown at present. To evaluate the effect of raised concentrations of IGF-I receptors on adrenocortical cell growth, we stably transfected the cDNA of the intact human IGF-I receptor into the mouse adrenocortical tumour cell line Y1. Overexpression of IGF-I receptors in Y1 adrenocortical tumour cells was able to induce IGF dependent cell growth and to antagonise the antiproliferative effect of adrenocorticotrophin (ACTH) in vitro. Furthermore, we assessed the influence of IGF-II on in vivo adrenal growth and function in transgenic PEPCK-IGF-II mice, which postnatally overexpress IGF-II. In these mice, postnatal overexpression of IGF-II induced a significantly increased adrenal weight and raised corticosterone serum values, presumably through a paracrine mitogenic effect of IGF-II on adrenocortical fasciculata cells (Weber *et al. Endocrinology* 1999;140:1537). It therefore seems evident that high local levels of IGF-II in combination with raised IGF-I receptor concentrations would represent a significant growth advantage of the adrenocortical carcinoma cell and thus could contribute to the highly malignant phenotype of this rare type of cancer. However, the fact that the described PEPCK-IGF-II mice did not develop

macroscopically obvious tumours over a period of 18 months suggests that IGF-II overproduction by itself is not sufficient for malignant transformation, and that additional factors are required for tumorigenesis. Recently, increased concentrations of IGF binding protein 2 (IGFBP-2) have been observed in human malignancies including adrenocortical carcinomas (Boulle *et al. J Clin Endocrinol Metab* 1998;83:1713). To elucidate the functional consequences of adrenocortical IGFBP-2 overexpression, we have stably transfected the cDNA of murine IGFBP-2 in Y1 mouse adrenocortical tumour cells. Overexpression of IGFBP-2 was associated with enhanced cell proliferation and colony formation, which was independent of exogenous IGFs (Höeflich *et al. Cancer Res* 2000;60:834). These data suggest that raised concentrations of IGFBP-2 might contribute to the highly malignant phenotype of adrenocortical cancer by a so far unknown, presumably IGF independent, mechanism.

#### **Abstract 56. The IGF/IGFBP system in CNS malignancy**

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Insulin-like growth factor I (IGF-I) and IGF-II are involved in the regulation of brain development and are thought to play a pivotal role in the proliferation of brain tumours. The expression of IGF-I, IGF-II, and the type I and type II IGF receptors were studied in a panel of 30 glioma cell lines by PCR analysis. PCR analysis revealed signals in 19 of 28 cell lines for IGF-I, 27 of 30 for IGF-II, 19 of 28 for the IGF-I receptor (IGF-IR), and 22 of 28 glioma cell lines for IGF-IIR. In addition, the secretion of IGF peptides was analysed by radioimmunoassay. Whereas immunoreactive IGF-I was below the level of detection, immunoreactive IGF-II values ranged from 125 to 5000 ng/ml supernatant. IGF receptor status and binding characteristics were established by Scatchard analysis. Proliferation assays showed different effects of IGFs and IGF analogues on the proliferation of these cell lines. Des-(1-3)IGF-I showed an unexpected inhibitory activity on glioma cell proliferation.

Crosslinking studies showed both cytoplasmic (CYT) and membrane bound (MB) IGFBPs as well as IGF-IIR proteins. MB IGFBPs with a molecular mass of 44, 50, and 50–60 kDa were found, whereas CYT IGFBPs did not exhibit the latter species. We have demonstrated the presence of IGFs and their receptors in a subgroup of glioma cell lines and suggest that the IGF system is involved in tumour growth regulation in vitro. IGFs may serve as progression factors upregulating growth in gliomas, and as survival factors blocking a common late intracellular apoptosis pathway.

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## Abstracts

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