IGF-II dependent autocrine growth in cell lines derived from renal tumours of childhood

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Abstract

Aims—To determine the role of insulin-like growth factors (IGF) in the proliferation of tumour cells, by studying the mitogenic response to IGFs of three cell lines of differing phenotype established from both malignant rhabdoid and Wilms tumour, representing a range of cell types (GOS 4, G401, and T3/73).

Methods—Production of IGF-II and IGF-I was measured by radioimmunoassay, and the presence of IGF binding protein complexes was observed by gel exclusion chromatography. Following growth analyses in serum-free media to ascertain the dependence of the cell lines on exogenous IGFs, the generation of autocrine growth was measured by a density dependence assay of proliferation in culture. Receptors were measured by radioligand cross linking and autocrine growth through these receptors assayed by the use of blocking antibodies.

Results—While GOS 4 and G401 were able to proliferate in serum-free medium over a period of 5 d, T3/73 showed an absolute dependence on IGFs added daily at 1–10 ng/ml. Plating at clonal density showed that cell growth was directly density dependent in serum-free medium. The serum independent proliferation of G401 and GOS 4 was blocked by the addition of an antibody to the type 1 IGF receptor (α -IR3) suggesting that the effects of autocrine factors are mediated through type 1 IGF receptors. S1 nuclease protection analysis indicated that all three cell lines produced significant amounts of mRNA derived mainly from the P3 IGF-II promoter, but transcripts for IGF-I were undetectable. Radioimmunoassay of IGFs from conditioned media showed that all the lines made assayable IGF-II (8.6, 8.4, and 6.1 ng/ml/24 h/10⁶ cells for GOS 4, G401, and T3/73 respectively). The presence of species consistent with both type 1 and type II IGF receptors was demonstrated using radioligand binding to cell membranes followed by cross linking.

Conclusions—Autocrine IGF-II may contribute to the serum independence of GOS 4 and G401 cells, whereas T3/73 may depend on exogenous IGF-II for proliferation.

Both Wilms tumour and renal malignant rhabdoid tumours are believed to be derived from aberrations in the genesis of normal differentiated kidney tissues during development.¹ Wilms tumour is an embryonal neoplasm of childhood which is thought to originate in delayed or abnormal differentiation of cells from the metanephric blastema,² while malignant rhabdoid tumour, originally classified as a rhabdomyosarcomatoid variant of Wilms tumour, is a very aggressive tumour believed to arise from more primitive mesodermal precursors.³ While Wilms tumour suppressor gene (WT-1) and other loci have been implicated in Wilms tumorigenesis, this gene codes for a zinc finger containing transcription factor, and it has therefore become of great interest to look for factors downstream of WT-1, in which altered regulation may cause the growth aberration characteristic of this tumour. One candidate factor is insulin-like growth factor II (IGF-II). IGF-II is a 67 amino acid peptide hormone, a member of the insulin related family of peptide growth factors which includes IGF-I, relaxin, and insulin itself. The biological actions of insulin, IGF-I, and IGF-II are mediated through at least three receptors and modulated by the action of a family of six IGF binding proteins, which may act either to inhibit or to potentiate the action of the peptide hormones (for general reviews see ⁴). It is interesting to note that a common cytogenetic lesion in malignant rhabdoid tumours is between D11S12 and TH at 11p15,⁵ a region also thought to be involved in the overgrowth and tumour predisposing Beckwith-Wiedemann syndrome, often associated with IGF-II overexpression.⁶

IGF-I and IGF-II are thought to be pivotal in embryonic growth and differentiation, including the development of the metanephric kidney.⁷ IGF-II is expressed at high levels in the metanephric blastema during nephrogenesis and has been shown to be important in blastemal cell growth in kidney organ culture.⁸ After birth, levels of IGF-II transcription drop 50–100 fold. Examination of the expression of the IGF genes in a variety of pathological processes⁹ indicates that kidney IGF-II is only increased in embryonal nephroblastoma (Wilms tumour) where the gene can be massively activated to levels only seen during development.^{10–12} This is associated with tumour cells in the kidney rather than existing normal adult cells. Increased transcription is not only seen in tissues characteristic of fetal expression (blastema) but also ectopically in the tumour derived nephrogenic epithelia.^{13–15} This observation has led to the hypothesis that IGF-II

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is responsible for the initiation or support of tumour cell growth. However, evidence implicating the inappropriate expression of IGF-II in a direct causative role is ambiguous. On the one hand IGF-II is normally suppressed by the Wilms tumour suppressor gene, WT-1.¹⁶ In tumours, however, mutation of WT-1 often accompanies induction of IGF-II expression. In addition the genomic imprint of IGF-II is also often lost in Wilms tumour, leading to biallelic expression and a doubling of transcript template.¹⁷

However, immunoassayable IGF-II is not always seen in Wilms tumour, and there is substantial evidence for failure to translate IGF-II mRNA.^{18,19} Inactivating mutations in IGF-II have been noted in Wilms tumour which would not be expected if there was strong selective pressure for IGF-II overexpression.²⁰ Release of the genomic imprint of IGF-II is seen in many different tumours,²¹⁻²³ but at the moment it is difficult to see how doubling of available template should lead to a 50- to 100-fold increase in transcription. It may be that loss of imprint is a tumour epiphenomenon rather than an initiating mutation. Recently, evidence from overexpression of IGF-II in transgenic mice has not shown rapid tumour induction, and tumours are only found after considerable latency.²⁴

We have tried to approach the question of the role of IGF-II in tumours by examining the production and response to IGF-II in cell lines derived from childhood kidney tumours. Three cell lines were selected which show distinct characteristics. G401²⁵ is a highly malignant epithelioid tumour, used much in the study of tumour suppression by WT-1. Garvin *et al.*²⁶ have suggested that it is most close in phenotype to malignant rhabdoid tumour, on the basis of the study of tumour cells passaged through a nude mouse. However, selection for higher grade malignancy by *in vivo* passage cannot be ruled out. GOS 4 was derived from a primary rhabdomyosarcomatoid Wilms tumour, later reclassified as malignant rhabdoid tumour, and gives rise to characteristic small cell tumours in xenograft culture. In culture the cells are mainly cytokeratin negative as might be expected for more primitive nephrogenic stem cells^{27,28}; however, cytokeratin positive cells arise in the clonal population sporadically (Schofield PN, unpublished data). The cells are N-CAM negative and express desmin weakly. T3/73 was established from a primary Wilms tumour with heterologous differentiation²⁹ and is much more mesenchymal in morphology, being 95% desmin positive and only 5% cytokeratin positive. It has never been shown to give rise to tumours in nude mice even at high inocula.

We show here that G401 and GOS 4 can grow in serum-free media through the generation of autocrine IGF-II. T3/73, however, makes insufficient IGF-II to complete an autocrine loop successfully. These results suggest that production of IGF-II by populations of cells in malignant rhabdoid tumours may be responsible for tumour cell proliferation or survival and also indicates that paracrine mech-

anisms may act on responsive components in Wilms tumour which do not necessarily over-express IGF-II.

Methods

CELL CULTURE

T3/73²⁹ was the kind gift of Dr Shant Kumar, Christie Hospital, Manchester; G401 was obtained from the ATCC. The line of G401 used in this experiment was cloned immediately before use in the experiments. Four separate clones behaved identically in growth assays and one was selected for further characterisation. GOS 4 was the gift of Dr John Cowell, Institute of Child Health, London. All lines were routinely cultured in a 50:50 vol/vol mix of Hams F12/ α modified Eagle medium (α /Ham) supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin and streptomycin as described in Biddle *et al.*³⁰ Serum-free medium consisted of the above without FCS and supplemented with 10 μ g/ml iron loaded human transferrin (Behring) as described in Heath.³¹

GROWTH ASSAYS

Serum-free growth assays were carried out essentially as described in Biddle *et al.*³⁰ Briefly, cells were washed in phosphate buffered saline (PBS) and harvested by treatment with trypsin/versine and chick plasma³² counted in a haemocytometer and plated at an appropriate density in 60 mm Primaria dishes (Falcon, Becton-Dickinson). For routine assays this was usually $1-3 \times 10^5$ per dish. The plating medium was α /Ham supplemented with 10% FCS pre-equilibrated at 37°C in 5% vol/vol CO₂ for 6 h previously. After attachment overnight, plates were washed five times in equilibrated serum-free medium and finally 5 ml of serum-free medium was added to each dish. Cells in three dishes were trypsinised and counted immediately to assess starting cell numbers. The range of cell numbers in the triplicate dishes did not exceed 10% of the mean cell count. Twenty four hours later cells were again counted and the medium changed. Test growth factors were then added to each treatment group in triplicate. Cell numbers were then counted on each successive day for 5 d at the same time of each day in triplicate.

Antibody inhibition studies were carried out according to the same protocol. Antibody α -IR3³³ was purchased from Cambridge Bioscience and added daily at 0, 1, 2, and 5 μ g/ml to serum-free cultures of cell lines. After 4 d of successive additions, triplicate plates were trypsinised and counted in a haemocytometer.

CLONAL ASSAYS

Cells were plated out as above at 5×10^3 , 1×10^4 , 2.5×10^4 , 5×10^4 , and 1×10^5 per dish in triplicate exactly as described above. After attachment for 24 h in 10% FCS, plates were washed and medium replaced with α /Ham.

At the end of day 5 cells were fixed in cold 3:1 methanol/acetic acid and colonies stained

in Giemsa stain. Scoring of colonies classed them into one of the following bins depending on cell number: 1, 2, 3–5, 6–10, 11–20.

GROWTH FACTORS

Recombinant human IGF-I and IGF-II were both gifts of Dr Anna Skottner, KaBi Pharmacia. Solutions were prepared according to Biddle *et al.*³⁰ and added on successive days to culture dishes. Control dishes received a dose of vehicle (BSA, 50 µg/ml) at the same concentration as that contained in the experimental dishes.

NUCLEASE PROTECTION ASSAYS

Nuclease protection assays were carried out on cell and tissue total RNA prepared according to Chirgwin *et al.*³⁴ The probe used was derived from IGF-II cDNA Hep 5 as described in Schofield and Tate.¹² The probe was cut internally with SallI, which cuts in the second coding exon of IGF-II, and end labelled by filling with Klenow fragment of DNA polymerase.

Following heating at 80°C for 10 min to denature the probe, 10 µg of total RNA were hybridised to excess 5' end labelled double stranded probe in a 10 µl reaction containing 80% formamide, 40 mM Pipes, pH 6.4, 0.4 M NaCl, and 1 mM EDTA under paraffin oil at 52°C overnight. Subsequently the reaction was placed on ice and 300 µl of ice cold S1 buffer [30 mM sodium acetate (pH 4.6), 4 mM zinc sulphate, 0.28 M NaCl, and 5% (vol/vol) glycerol containing 15 µg of sonicated denatured salmon sperm DNA as carrier], and 60 units S1 nuclease (BRL) were added. After incubation at 30°C for 1 h the reaction was terminated by the addition of 100 µl 15 mM EDTA, pH 7.9, containing 30 µg tRNA carrier, followed by ethanol precipitation. Reaction products were separated on denaturing acrylamide gels, using an end labelled 123 bp ladder (BRL) or a sequencing ladder as markers.

RADIORECEPTOR CROSS LINKING

Radioreceptor cross linking was carried out as described in Biddle *et al.*³⁰ Unlabelled competitors used were 2 µg/ml IGF-II and 5 µg/ml insulin (Sigma, RIA grade). Markers were Amersham Rainbow protein markers and labelled tracer [Amersham International; ¹²⁵I-iodotyrosyl IGF-I (specific activity 2000 Ci/mmol)].

MEASUREMENT OF IGF-I AND IGF-II PEPTIDES

Collection of conditioned medium was made in the same way as described in Schofield *et al.*³⁵ 2 × 10⁶ cells were plated in T175 tissue culture flasks (Nunc, Gibco) and when the cells approached but did not reach confluence they were washed five times in prewarmed medium, after which 15 ml of serum-free medium was added; after 18 h, medium was

aspirated, centrifuged at 180 × g for 20 min to remove debris, and frozen at –70°C.

An aliquot of 10 ml conditioned medium derived from Wilms tumour cell lines as described above was freeze dried, reconstituted in 250 µl of 2 M acetic acid, and loaded onto a Sephacryl S-200 HR column (30 × 1 cm; Pharmacia) equilibrated with 1 M acetic acid, pH 4.0. Fractions (1 ml) were collected and aliquots of 250 µl freeze dried. Fractions equivalent to a K_d between 0.82 and 0.95 (for IGF-I and IGF-II) and 0.69–0.77 (for high molecular weight forms) were analysed by radioimmunoassay. IGF-I was measured as described³⁶ using recombinant IGF-I (KaBi Pharmacia) as standard. Cross reaction of IGF-II was less than 1%. IGF-II was measured similarly using a monoclonal antibody to IGF-II (MAS 324, clone S1-F2) (Seralab) and recombinant IGF-II (KaBi Pharmacia AB) as standard. Each vial contained 10 µg of IgG and was reconstituted in 1 ml of buffer. The initial dilution of the stock solution added to the assay was 1:6000. Cross reactivity for IGF-II in the IGF-I assay was 1.4%. Iodinated human IGFBP-1 purified from human plasma was the kind gift of Dr D Morrell.

Results

PROLIFERATION OF CELL LINES AT CLONAL DENSITY

In order to examine the potential for autocrine growth of the cell lines under investigation we assessed the effects of cell density on clonal proliferation. Should an autocrine factor be rate limiting on cell division or survival we would expect to see a density dependence of proliferation. Cells were plated at differing clonal densities as described in Methods, allowed to grow for 7 d after plating, with the medium changed to serum-free after day 1. At the end of the experiment the colonies were well defined, with cells making close contacts in all three cell lines. The results are shown in fig 1. Each column represents the mean of three separate determinations.

In the assay carried out, cells were plated in 10% FCS to reduce variability in plating efficiency, which is often markedly density dependent. Differences in plating efficiency are reflected in the overall numbers of colonies in all categories in fig 1. The overall number of colonies was greatest for G401, followed by GOS 4 and then T3/73. This differential is composed of the intrinsic cloning efficiency and cell survival. Comparison of the distribution of clone sizes within each data set at the end of the experiment indicates that in the case of T3/73 most of the cells failed to divide at all, and a small number divided twice during the course of the experiment. More cells survived and divided with increasing cell density. This trend was marked in the case of GOS 4. While there was still a significant percentage of cells which did not divide, many more did, and at high plating density these were capable of forming large colonies. The most extreme effects were seen with G401 where all of the cells which survived plating divided more than twice and

Figure 1 Dependence of serum-free growth on plating density. Cells were plated out at 5×10^3 , 1×10^4 , 2.5×10^4 , 5×10^5 per dish in triplicate, exactly as described in Methods. After plating in serum and allowing 24 h for attachment, plates were washed and medium replaced with serum-free medium. At the end of day 6, cells were fixed and colonies stained in Giemsa stain. A = T3/73; B = GOS 4; C = G401. Scoring of colonies classed them into one of the following categories, depending on cell number within the colony: 1, 2, 3–5, 6–10, and 11–20. The plot compares three variables: initial plating density (cell density), colony formation (colonies per category), and colony size (category). Accumulation of colonies in the rear of the plot implies an effect of initial plating density on colony number. Accumulation in the extreme right hand rear of the plot indicates growth cooperativity, and in the extreme left rear of the plot, lack of such cooperativity. The effects of numbers of cells plated on plating efficiency would be expected to increase the number of colonies with increasing input of cells without increasing colony size should growth cooperation not be present. Both trends are visible (see Discussion).

many gave rise to large colonies. Again there was a marked effect of initial plating density, the largest colonies being seen at the highest plating densities (note that there were no colonies in the 2–3 and 3–5 categories in fig 1C).

These data suggest that cross feeding of some factor, previously termed “metabolic co-operation” exists to a greater or lesser degree in all of the cell lines examined, being most marked in the case of G401.

IGF DEPENDENCE OF CELL GROWTH

Wilms tumours have previously been shown to express inappropriate amounts of IGF-II, and it was therefore pertinent to examine whether the effects of IGF-II might augment the serum-free growth of these cell lines, and whether their basal proliferation seen in serum-free medium was mediated by autocrine IGFs.

Growth assays were set up as described in Methods. Daily additions of IGF-II at 1 or 10 ng/ml for 5 d (fig 2) showed that G401 cells grew at the same rate in serum-free medium, and almost matched that in 10% FCS. IGFs are therefore not rate limiting for G401 population growth. In the case of GOS 4, again IGF-II was unable to support the growth of the cells more than seen with serum-free medium. Initially the growth was indistinguishable from that in FCS but reached a plateau at 5 d, suggesting that another factor had become rate limiting. This may either be the lack of a specific factor or a build up of toxic agents in the serum-free medium. In the light of the FCS kinetics the former is most likely. T3/73 on the other hand showed an absolute dependence on exogenous IGF-II, 1 ng/ml IGF-II per day being sufficient to support growth to the same extent as 10% FCS. As noted in the clonal growth experiments, little proliferation was seen in serum-free medium, but some cells were able to survive for 5 d after FCS withdrawal.

Addition of an antibody which blocks the ability of the type I IGF receptor to mediate an IGF-I or II signal (fig 3) caused a reduction in serum-free cell growth of G401 and GOS 4 over 4 d in serum-free medium. In the case of G401 a dose dependency of inhibition was clearly seen, being maximal between 2 and $5 \mu\text{g/ml}$. More antibody was required to suppress GOS 4 growth, but this was proportionately more effective. A small and statistically non-significant rise in cell numbers was seen with T3/73. Positive agonist effects have been noted before with this antibody, and we suggest that this slight upward trend is the result of receptor cross linking.³⁷

MEDIATION OF AUTOCRINE EFFECTS THROUGH THE TYPE I IGF RECEPTOR

The previous experiments suggest that the basal level proliferation seen in GOS 4 and G401 is due to autocrine stimulation of the type I IGF receptor. In order formally to demonstrate the presence of this receptor on the surface of these three cell types, we carried out a receptor/radioligand cross linking experiment as described in Methods. The results are shown in fig 4. In all three cell lines three species are cross linked to iodinated IGF-I: two with an M_r around 270 kDa and one with M_r of approximately 130 kDa. These species are consistent with the heterogeneously glycosylated type

Figure 2 Effects of IGF-II on growth in serum-free medium. Cells were plated out in 5 cm tissue culture dishes as described in Methods, at 2×10^5 cells per dish in 10% fetal calf serum. After attachment for 24 h, plates were washed and medium replaced with serum-free medium (α /Ham), α /Ham containing 10% FCS, α /Ham containing 1 ng/ml IGF-II, and α /Ham containing 10 ng/ml IGF-II. Additions of growth factors were made daily for 6 d and on each day triplicate dishes were counted in a haemocytometer. The results given are a typical example of three replicate experiments. A = G401; B = GOS 4; C = T3/73.

Figure 3 Effects of anti-receptor antibody on serum-free growth. Cells were plated out at 2×10^3 per well in α /Ham containing 10% fetal calf serum in 24-well plates (Nunc). Following attachment, plates were washed and medium replaced with α /Ham alone plus antibody α -IR3 in 50 μ g/ml bovine serum albumin, at 0, 1, 2, and 5 μ g/ml. Additions were continued for 4 d, then triplicate plates were trypsinised and cells counted in a haemocytometer. A = G401; B = GOS 4; C = T3/73; FCS = numbers of cells after 4 d in presence of fetal calf serum. *, **Significant decrease in cell numbers v serum-free control, $p < 0.05$, $p < 0.01$ (Student *t* test).

Figure 4 Cross linking of IGF receptors to radiolabelled IGF-I. Membrane protein (45 μ g), prepared according to Methods, was incubated with tracer (2.5 mM) iodinated IGF-I in the presence of 2 μ g/ml IGF-II competitor, 5 μ g/ml unlabelled insulin competitor, or buffer alone. Following cross linking with disuccinimidyl suberate, membranes were loaded onto a denaturing sodium dodecyl sulphate (SDS) polyacrylamide gel [3–14% (wt/vol) gradient] following denaturation of proteins in the presence of SDS and β -mercaptoethanol (2% vol/vol). Markers used were Amersham rainbow markers with M_r indicated. Gels were dried and exposed to Kodak X-AR film for two weeks.

II IGF receptor at 270 kDa and the α chain of the type I receptor at about 130 kDa.^{30,37} Insulin was surprisingly ineffective at competing with IGF-I for the type I receptor, but this could be seen clearly with saturating concentrations of IGF-II (and IGF-I; data not shown), as expected.

SYNTHESIS OF IGF-II BY WILMS TUMOUR AND CELL LINES IN CULTURE

The above experiments indicate that GOS 4 and G401 are both capable of autocrine serum-independent growth mediated by factors acting through the type I IGF receptor. We wished to establish whether these cell lines synthesised IGF-I or IGF-II. S1 nuclease protection assays using a cDNA probe to human IGF-II showed the presence of hybridisable RNA for IGF-II in all three cell lines at markedly differing concentrations (fig 5). Transcripts representing transcription from the P3 IGF-II promoter predominated in T3/73 and G401 (species A at 359 bp), and could also be detected in two of three triphasic primary Wilms tumours and first trimester human fetal kidney RNA. GOS 4 also expressed RNA from this promoter but at markedly reduced levels. Two other species were apparent at 127 bp (C) and 287 bp (B). The former represents a discontinuity between probe and target at the position of alternative splicing in IGF-II,^{12,38} and the latter a discontinuity at the exon 7 5' border. This could reflect transcripts derived either from the P2,

Figure 5 Synthesis of IGF-II mRNA by Wilms tumour and tumour cell lines. Total RNA (10 µg) was subjected to S1 nuclease protection assay as described in Methods, using a cDNA probe end labelled at the Sal I site in exon 8 (representes as "coding" in the figure) and extending into exon 5 (represented as "fetal exon"), which is associated with the major fetal promoter of IGF-II. Protected fragments were apparent at C: 127 bp, B: 287 bp, and A: 359 bp. Size markers were a 123 bp ladder from BRL. Uncut probe can be seen at the top of the gel. "Probe" refers to a sample with no test RNA added, in which the probe was completely degraded by S1 action. A: fully protected RNA arising from the P3 promoter; B: discontinuous at the 5' end of exon 8; C: discontinuity at the site of the internal coding sequence alternative splice. AmWh, BeMa, and BeOc are primary triphasic Wilms tumours; the fetal kidney sample was from a first trimester human embryo (8 weeks gestation); the adult kidney was from an infant of 4 years.

Figure 6 Radioimmunoassay of IGF-II in conditioned medium from Wilms tumour cell lines. Conditioned media were subjected to either an acid (pH 4) or a neutral (pH 7.4) gel filtration column as described in Methods; 1 ml fractions were freeze dried and IGF-II was radioimmunoassayed. The positions of IGF-I/IGFBP-1 complexes are marked with an arrow. Radiolabelled IGF-II was used as a marker for 7.5 kDa IGF-II. K_d represents the fractional volume eluted from the column.

P4, or P1 promoter.³⁹ The data show the presence of IGF-II transcripts which are capable of giving rise to IGF-II protein. Radioimmunoassay of conditioned medium from these cell lines indicated that this IGF-II RNA was translated. IGF-I radioimmunoassay showed insufficient material to be distinguished from the background of the assay (data not shown).

Figure 6 shows radioimmunoassay of IGF-II from conditioned medium separated over a neutral or acidic gel filtration column. Most of the IGF-II present was found at a column fraction of K_d 0.2 co-eluting with radiolabelled IGFBP-1, indicating that most if not all of the IGF-II produced was complexed to binding proteins in conditioned medium. When acid-treated to strip the IGF-II from its binding protein, component 7.5 and 12 kDa species of IGF-II were apparent in medium from GOS 4 and G401, but with less detectable in T3/73. Between 11% and 15% of all IGF-II in GOS4 and G401 was 12–15 kDa in size. Summed over the collection period the quantity of total

IGF-II species was 8.6, 8.4, and 6.1 ng/ml/24 h/10⁶ cells for G401, GOS 4, and T3/73 respectively.

Discussion

We have shown that three cell lines derived from childhood renal tumours all possess type I and type II IGF receptors. The type I receptor is the route by which two of these lines, GOS 4 and G401, are able to complete an autocrine growth loop dependent on IGF-II. All three cell lines make significant IGF-II peptide and it is unlikely that the amount is insufficient in the case of T3/73 to drive autocrine proliferation. The failure to do so may either be because there is an additional rate limiting requirement for factors other than IGF-II, or possibly because of the synthesis of inhibitory binding proteins. Comparison between IGF-II mRNA synthesised and assayable peptide suggests that either translational control is exerted in the case of T3/73, where the discrepancy is most apparent, or that the peptide derived from these cells has a shorter half life in conditioned medium. Most of the IGF-II produced by these lines is complexed to IGF binding proteins. Whether this is all available to cellular receptors is not addressed by these experiments, but abrogation of autocrine growth by α -IR3 strongly suggests that at least some of it is accessible. The identity of these binding proteins is currently under investigation. We have already reported enhanced levels of IGFBP-2 in serum of patients with Wilms tumour,⁴⁰ but in this instance it is not clear whether the IGFBPs are of tumour or host origin. The molecular size of the complexes when assayed on a neutral gel exclusion column is consistent with IGFBP-2 involvement. IGFBP-2 is most often an inhibitor of IGF action,⁴¹ but recent evidence suggests that it may also act to enhance bioactivity in some cell lines, and it is frequently expressed in both primary Wilms tumour and cultures derived from this tumour.⁴² High molecular weight species (12–15 kDa) of IGF-II, representing partially processed propeptides, are found in adult serum (see⁴⁰) and make up the majority of assayable IGF-II in many fetal tissues.⁴³

The synthesis of type I IGF receptors and IGF-II by Wilms tumour cells and tumours has already been reported and implicated in tumour growth.^{44,45} Gansler *et al*⁴⁶ have reported that infusion of high levels of purified anti-IGF type I receptor antibodies inhibits the growth of tumour fragments and primary cell lines transplanted into nude mice, but this conflicts with the findings of Little *et al*,⁴⁷ who were able to show that IGF-II expression was selected against during tumour passage as a xenograft and that it makes use of unusually high levels of antibody delivery. Nevertheless, receptors are present in primary tumours⁴⁴ and the type I receptor gene has been shown to be controlled by WT-1 such that mutation of WT-1 leads to increased expression of the type I receptor.⁴⁸ The importance of this in natural tumorigenesis remains to be elucidated but it is significant

that deletion of the type I IGF receptor prevents *in vitro* transformation by SV40 T antigen.⁴⁹ We were surprised to find that insulin competed poorly with IGF-I receptors. This may reflect the presence of atypical type I receptors or a high concentration of high affinity binding sites for insulin on these cell lines. Further experiments are needed to clarify this result.

Both IGF-I and IGF-II have been implicated in the unscheduled proliferation seen in benign hyperplastic and malignant cell growth in a wide range of tumours (reviewed in^{50,51}). Autocrine growth loops have been demonstrated in culture for rhabdomyosarcoma,⁵² small cell lung carcinoma,⁵³ breast carcinoma,⁵⁴ pheochromocytoma,⁵⁵ and neuroblastoma.⁵⁶ IGF-II dependent paracrine activity has also been postulated for some breast tumours.⁵⁷

A great deal of attention has recently been paid to the control of IGF-II imprinting and its potential involvement in Wilms tumour. Under normal circumstances IGF-II is only expressed from the paternally inherited IGF-II allele.⁵⁸ Several groups have now shown that in a range of tumours including Wilms tumour the genomic imprint is lost, leading to biallelic expression.^{17,59,60} However, two of these studies noted that loss of imprint was only associated with doubling of the very high levels of expression of IGF-II characteristic of most Wilms tumours,¹⁰ and it is difficult to reconcile the relatively small increase in transcript levels expected from reactivating the maternal allele with the huge expression levels seen in Wilms tumour.

In vivo evidence is not entirely consistent with a role for IGF-II in tumorigenesis. Where IGF-II expression has been examined at both the mRNA and protein level in specific tumours, high levels of mRNA are often not associated with high levels of tissue or secreted IGF-II protein, and high levels of antisense transcripts have been detected in several Wilms tumours associated with lack of peptide,^{18,19} suggesting negative post-transcriptional regulation of protein production. Such post-transcriptional regulation is seen in the embryo⁶¹ and is characteristic of the combination of tissues and predominant promoter usage. Paradoxically, in a model system, forced expression of IGF-II has been shown to prevent growth of sarcomas grafted into nude mice.⁶²

Our results strongly support the contention that in some circumstances expression of IGF-II may assist cell proliferation or survival. It is important to note that the ability to execute autocrine growth in culture correlates with the ability to form tumours in nude mice. Whether this is also rate limiting on tumour growth *in vivo* is difficult to assess, and IGF-II expression may be a tumour epiphenomenon, being a characteristic of the cell type in which the tumour originates. Such expression may be important in the establishment of cultured cell lines, but it is also seen *in vivo*, suggesting that it may give a selective advantage at some stage of tumorigenesis. The observation that T3/73 is IGF dependent but unable to complete an autocrine loop itself suggests that if similar cells are present during tumour formation or

differentiation they may depend on paracrine stimulation. Dowdy *et al.*⁶³ using a different clone of G401, were able to show that serum independence of proliferation correlated with tumorigenicity of lines carrying a tumour suppressing fragment of chromosome 11, microcell mediated chromosome transfer preventing both tumour formation in nude mice and serum-free growth. This group was able to show that the parental cell clone could provide a factor in conditioned medium which allowed serum-free growth of the suppressed cell line, but was unable to identify this factor. While transferrin clearly had a beneficial effect on growth, these investigators were unable to show differences in transferrin expression between clones. The data presented here suggest that the factor in tumorigenic G401 medium was IGF-II. This is consistent with the properties of the cell lines examined here and suggests that IGF-II expression is a prerequisite for xenograft growth. It is possibly significant that GOS4, a renal MRT cell line, and G401, postulated to be such, are both more aggressive tumours and autocrine for IGF-II.

The expression of IGF binding proteins by these cell lines suggests that before we can accurately define the potential role of IGFs in Wilms tumour and related cancers we must take into account factors modulating their activity. Both IGF-I and IGF-II have been shown to act as survival factors or anti-apoptotic factors in a variety of systems, including human teratocarcinoma⁶⁴⁻⁶⁶ and have been implicated in SV40 T induced insulinomas.⁶⁷ Should this be the major role of IGF-II then further factors may be needed to act in concert to generate the neoplastic process.

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