Insulin-like growth factors I and II induce cell death in Wilms’s tumour cells

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Abstract

Aim—To study the effects of insulin-like growth factors (IGFs) on the growth phenotype of a Wilms’s tumour cell line (WCCS-1).

Methods—WCCS-1 cells were cultured in vitro and exposed to IGF-I and IGF-II, as well as their antagonists, IGF binding protein 2 and the type I receptor blocking antibody IGF-IRa. The effects on proliferation and cell cycle parameters were assayed by assessing cell numbers, autoradiography after labelling with tritiated thymidine, and flow cytometry after double staining with fluorescein isothiocyanate (FITC) labelled annexin V and propidium iodide.

Results—The addition of IGF-I as well as IGF-II in physiological doses induced cell death in Wilms’s tumour cells. Cell numbers decreased most dramatically on the fifth to sixth day after growth factor addition. The occurrence of apoptosis as well as necrosis was confirmed by annexin-V staining of cell cultures. S-phase indices were comparable, irrespective of whether the cells were exposed to IGFs or not, which suggests that WCCS-1 cells undergo cell death at random during the cell cycle rather than from the prereplicative phase. To exclude any influences of the IGF binding proteins (IGFBPs), all results were repeated with Des(1-3)IGF-I, which is unable to bind to any of the IGFBPs. However, this peptide was equally potent in inducing cell death. Finally, the addition of IGFBP-2 or the type 1 receptor blocking antibody IGF-IRa partly abrogated the death inducing effects of IGF-I and IGF-II.

Conclusions—Insulin like growth factors induce cell death—apoptosis as well as necrosis—in cultured Wilms’s tumour cells. Furthermore, it is proposed that this effect is mediated by the type 1 receptor.

Keywords: insulin-like growth factor I; insulin-like growth factor II; Wilms’s tumour; apoptosis

Wilms’s tumour is a childhood neoplasm that is believed to develop when multipotent kidney blastemal cells fail to differentiate and continue to proliferate after birth.1 The occurrence of sporadic as well as hereditary variants of Wilms’s tumour and the early age of bilateral kidney tumour onset suggested that Wilms’s tumour development when a predisposing germ locus associated with Wilms’s tumour led to the cloning and characterisation of the gene, WT1, which encodes a zinc finger DNA binding protein that functions as a transcriptional repressor.2,3 One of the first actions revealed for WT1 was its repression of the insulin-like growth factor II (IGF-II) gene. Wilms’s tumours are in general characterised by a high rate of expression of the IGF-II gene.3,4 IGF-II is known to be a ubiquitous and essential growth factor during embryogenesis,3,5 and acts as a survival factor in embryonic tumour cell lines.5–11 Taken together, these data suggested a growth regulatory loop whereby IGF-II supports either proliferation or survival and WT1 counteracts this by specifically suppressing IGF-II transcription. In this report we challenge the universal applicability of this concept. Wilms’s tumour cells grown in vitro respond to either of the IGFs by an increased rate of cell death. Even though growth inhibitory effects have been recorded previously for IGF-II,12 and IGF II can act as a tumour suppressor in vivo, we show for the first time that both IGFs can induce cell death—in one Wilms’s tumour derived cell line at least—rather than unequivocally acting as a survival factor for tumour cells.

Material and methods

CELL CULTURE

The WCCS-1 cell line was maintained and flasks and wells for experimental purpose were prepared essentially as described by Talts et al.13 All tissue culture material was obtained from NUNC (Roskilde, Denmark) and GIBCO (through Life Technologies, Paisley, UK).

The serum free medium consisted of a 1/1 (vol/vol) mixture of α medium lacking nucleosides and deoxynucleosides and Ham’s F12 medium supplemented with 10 µg/ml of human transferrin preloaded with iron, in accordance with the manufacturer’s instructions. This medium is subsequently called α;Ham.

Two days before a growth experiment the medium over the stock cultures was replaced by α;Ham supplemented with 10% heat inactivated fetal calf serum (FCS). This procedure allowed the cells to adapt to the basal medium that was used in the serum free culture experiments. The cells were removed from the stock culture flasks by rinsing with phosphate buffered saline (PBS) and then briefly exposing the cells to a mixture of trypsin, versene, and chick plasma (TVP). The cells were taken up in α;Ham with 10% FCS, spun once, resuspended in α;Ham with 10% FCS, and counted in a Coulter counter (Coulter Electronics Ltd, Bedford, UK).
In the subsequent cell culture experiments, the media were pre-equilibrated for at least six hours in an atmosphere of 5% (vol/vol) CO₂ in 95% humidified air at 37°C. The day before the experiment, the cells were plated out in 10 ml of α-Ham with 10% FCS at a density that varied between $1.4 \times 10^5$ and $3.0 \times 10^5$ cells/60 mm diameter Primaria dish (Becton Dickinson, San Jose, California, USA). The number of cells plated out varied between experiments but numbers were always comparable within each experiment. At the start of each experiment, the cells were rinsed twice in PBS and 10 ml of α-Ham was then added to each dish. During the next hour the dishes were briefly removed from the incubator and the various experimental compounds were added to the dishes. Two dishes were counted immediately after the PBS rinse to obtain a starting cell count, and on subsequent days the effect of the additives was measured by counting duplicate dishes in each treatment. For these counts, the cells were exposed to trypsin (0.125%, wt/vol) and EDTA (wt/vol) in PBS for up to 30 minutes to ensure complete detachment from the dish surface. When all cells had detached, any remaining trypsin was neutralised by adding soybean trypsin inhibitor. The cells were counted in triplicate in a Coulter counter. The range of the cell counts of the duplicate dishes rarely exceeded 8% of the mean cell count.

GROWTH FACTORS AND OTHER ADDITIVES

Recombinant IGF-I and IGF-II were purchased from British Biotechnology, UK. Modified Des(1-3)IGF-II was obtained from GroPep (Adelaide, Australia). Bovine IGF binding protein 2 (IGFBP-2) was purchased from GroPep, and the IGF-IR (1H7) antibody, which specifically recognises the IGF-I receptor was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). The lyophilised IGF-I and IGF-II samples were made up to stocks of 2 µg/ml by adding 0.5 ml of 0.1 M acetic acid to 10 µg, shaking every five minutes in a 37°C water bath, then adding 4 ml of PBS with 1% wt/vol crystalline bovine serum albumin (BSA; Sigma, Stockholm, Sweden), adjusting the pH to 7.0 with 0.1 M NaOH, and finally bringing the volume up to 5 ml with PBS/BSA. These stocks were aliquoted into Eppendorf vials and stored at −70°C until use.

The IGFBP-2 was dissolved in sterile filtered 10 mM HCl at a concentration of 100 µg/ml, snap frozen in liquid nitrogen, and stored until further use. The anti-IGF-IIR (1H7) antibody, which specifically recognises the IGF-I receptor type 1 subunit, and thereby inhibits binding of both IGF-I and IGF-II to this receptor was supplied in the form of 200 µg IgG dissolved in 1 ml of PBS with 0.1% sodium azide and 0.2% gelatin. This vial was kept at 4°C.

In all growth experiments, growth factor was added on a daily basis at the same concentration as stated in the tables and figures. Control dishes were supplemented with either 5% FCS or with a dose of BSA, at the same concentration as that used as a vehicle for the growth factor. This procedure ensured that the control dishes were exposed to the same degree of cooling and the same pH changes.
obtained during repeated experiments. In contrast, when the cells were grown in the absence of macromolecular additives, the increase in cell numbers was retarded during the first four to five days, but only after five days in the absence of serum or other growth promoting substances did cell numbers begin to decrease. When WCCS-1 cells were cultured in the presence of 30 ng/ml IGF-I or IGF-II, there was a conspicuous drop in cell numbers by the fourth day in culture. After five or six days, in the presence of IGF-I or IGF-II cell numbers were far below those seen in cultures grown in serum free medium (table 1). Even low concentrations of IGF-I or IGF-II (1 ng/ml medium) exerted an observable repressive effect on cell numbers. This suppression of cell numbers increased as the concentration of IGFs increased. Figure 1 shows the morphological appearance of the cells in the presence or absence of IGFs. It is clearly shown that the cellular morphology does not change with changing culture conditions. The cells retain their polygonal shape and exhibited a clear epithelial-like morphology.

To exclude any spurious effect exerted by any of the circulating IGF binding proteins (IGFBPs), we examined the effects of a modified IGF-I molecule (Des(1-3)IGF-I), which binds to the type 1 receptor with unaltered affinity but not to any of the IGFBPs. This modified growth factor was just as potent as IGF-I and IGF-II in causing a decrease in cell numbers. To pursue this issue further, we concomitantly exposed WCCS-1 cells to IGF-I or IGF-II and an excess of IGFBP-2. Table 2 shows that the presence of the binding protein abrogated the inhibitory action of IGF-I or IGF-II on cell numbers. Similarly, the effect of IGF-I or IGF-II on cell numbers could be counteracted by the presence of anti-IGF-IRá, which is an antibody that specifically recognizes the á subunit of the IGF type 1 receptor. However, it was unclear whether the addition of either of the IGFs forced cells to leave the cell cycle and to enter G0. To elucidate whether this was the case, we pulse labelled cells after 72 hours with tritiated thymidine, to obtain a measure of the proportion of cells traversing the cell cycle (table 3). It was found that, irrespective of culture conditions, the thymidine labelling indices varied only between 24% and 29%, which suggests that in each of these scenarios, the surviving WCCS-1 cells

<table>
<thead>
<tr>
<th>Day</th>
<th>SFM</th>
<th>10% FCS</th>
<th>IGF-I</th>
<th>IGF-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>165</td>
<td>165</td>
<td>165</td>
<td>165</td>
</tr>
<tr>
<td>4</td>
<td>204</td>
<td>410</td>
<td>136</td>
<td>192</td>
</tr>
<tr>
<td>5</td>
<td>189</td>
<td>586</td>
<td>111</td>
<td>103</td>
</tr>
<tr>
<td>6</td>
<td>175</td>
<td>612</td>
<td>86</td>
<td>79</td>
</tr>
</tbody>
</table>

Cells were seeded in αHam supplemented with 10% FCS on day 0. On day 1 each culture was rinsed and αHam with supplements (10% FCS, 30 ng/ml IGF-I or IGF-II) was added. To ensure a continuous supply of growth factors, serum or the appropriate growth factor was added daily. Cells were counted in triplicate on a Coulter counter on the days indicated in the table. Each figure is based on two different experiments and the data expressed as mean (SD).

FCS, fetal calf serum; IGF, insulin-like growth factor; SFM, serum free medium.

Figure 1. Morphological appearance of WCCS-1 cells grown for five days in (A) 10% serum, (B) serum free medium (SFM) (C) SFM + 30 ng/ml insulin-like growth factor I (IGF-I), and (D) SFM + 30 ng/ml IGF-II.
Cells were seeded in α Ham supplemented with 10% FCS on day 0. On day 1 each culture was rinsed and α Ham with the appropriate additives was added (10% FCS, 30 ng/ml IGF-I, 50 ng/ml IGFBP-2, 20 ng IGF-IR antibodies). Serum or the appropriate supplement was added daily to ensure a continuous supply in the cell cultures. Cells were counted in triplicate in a Coulter counter on the days indicated in the table. Each figure is based on two different experiments and expressed as mean (SD).

Table 2: The effect of IGF antagonists on cell numbers

<table>
<thead>
<tr>
<th>Day</th>
<th>SFM</th>
<th>10% FCS</th>
<th>IGF-I</th>
<th>IGF-I+</th>
<th>IGF-I+</th>
<th>IGF-I+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SFM+</td>
<td>SFM+</td>
</tr>
<tr>
<td>1</td>
<td>165 (28)</td>
<td>165 (28)</td>
<td>165 (28)</td>
<td>165 (28)</td>
<td>165 (28)</td>
<td>165 (28)</td>
</tr>
<tr>
<td>2</td>
<td>204 (49)</td>
<td>410 (99)</td>
<td>136 (38)</td>
<td>355 (71)</td>
<td>399 (87)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>189 (55)</td>
<td>586 (88)</td>
<td>111 (19)</td>
<td>381 (55)</td>
<td>312 (26)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>175 (42)</td>
<td>612 (109)</td>
<td>86 (59)</td>
<td>361 (39)</td>
<td>401 (98)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean (SD).

Cells were seeded at sparse density and grown in α Ham supplemented with 10% serum for 24 hours and then rinsed in phosphate buffered saline and exposed to the experimental media for 72 h. Between the 71st and 72nd hour, the cultures were fixed and subjected to autoradiography. The proportion of cells labelled with tritiated thymidine was determined by light microscopy and expressed as percentages to adjust for the large difference in total cell numbers seen in the different experimental situations.

Table 3: The effect of growth factor addition on DNA synthesis in WCCS-1 cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>% Labeled cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% serum</td>
<td>28.5 (7.4)</td>
</tr>
<tr>
<td>α Ham (SFM)</td>
<td>29.1 (5.6)</td>
</tr>
<tr>
<td>SFM + 10 ng IGF-I/ml</td>
<td>26.6 (8.1)</td>
</tr>
<tr>
<td>SFM + 10 ng IGF-II/ml</td>
<td>29.7 (6.0)</td>
</tr>
<tr>
<td>SFM + 10 ng Des(1-3)IGF-1/ml</td>
<td>27.0 (9.6)</td>
</tr>
</tbody>
</table>

Values are mean (SD).

IGF, insulin-like growth factor; SFM, serum free medium.

Discussion

The role of growth factors as multifunctional polypeptides is becoming increasingly diverse. Certain growth factors that normally support the proliferation of specific cells can act as harbingers of death when they bind to tumour cells. One early example of this role was demonstrated for the hepatocyte growth factor, which stimulates the proliferation of hepatocytes as well as endothelial cells and melanocytes, but possesses tumoricidal activity in a variety of other cell types.

This study has shown that the addition of the insulin-like growth factors IGF-I and IGF-II induces cell death in Wilms’ tumour cells cultured in vitro. There was also a slight qualitative difference between the two growth factors because IGF-I appeared to exert a preferential effect on the induction of apoptosis, whereas IGF-II addition appeared to yield a relatively larger necrotic cell population. At first sight this observation is puzzling, but there are several recorded examples of inverse interactions between IGF-I and IGF-II, and a possible explanation for their slightly different qualitative effects on cell death is that the two growth factors bind with different affinities to the two receptors—the signal transducing type 1 receptor and the endocytic type 2 receptor. However, it was shown that blocking of the type 1 receptor abrogated the effect of IGF addition on cell death, indicating a key role of this receptor in the IGF induced lethal pathways.

The biological role of the IGFs is becoming increasingly complex. Whereas it is generally accepted that they play a pivotal role in embryonic growth and development, most convincingly demonstrated by experimental disruption of the IGF-II gene in transgenic mice, their role in tumour development and growth is less well understood.

In many naturally occurring and experimentally induced tumours, the fetal IGF-II promoters are reactivated. This was first seen in a group of developmental tumours including hepatoblastoma, embryonal nephroblastoma (Wilms’s tumour), adenocortical carcinoma, and rhabdomyosarcoma. One feature that these tumours have in common is that the corresponding normal fetal tissues make large...
quantities of IGF-II mRNA and protein. By studying gene expression during fetal kidney formation it was concluded that the high expression of IGF-II by the blastema component of Wilms’s tumour reflects the corresponding expression in normal blastema during the developmental window in which the tumour is thought to arise. This evidence suggested for a long time that the growth and development of embryonic tumours was aided by an oncofetal paracrine loop in which IGF-II was a prime messenger. However, our study demonstrates that IGF-II (as well as IGF-I), rather than fulfilling their expected role as stimulants of cell proliferation and survival, in fact induced cell death in Wilms’s tumour cells in vitro.

This finding is in line with two other pieces of evidence. First, there is a notable report where excess concentrations of IGF-II decreased skin sensory innervation in transgenic animals, thereby indicating a potential growth inhibitory role of IGF-II on a normal tissue in vivo. Second, forced expression of IGF-II was found to prevent tumour formation in nude mice, which suggests a clear role of IGF-II as a tumour suppressor in vivo. Moreover, there are qualitative as well as quantitative differences in biological activity between bona fide IGF-II and larger variants that are sometimes produced by tumour cells.

This novel role of IGF-II as a tumour suppressor conforms with earlier genetic data. Spontaneous as well as hereditary Wilms’s tumour allele losses around the IGF-II locus (11p15.5) have been described. On the other hand, imprinting of the IGF-II gene, which by itself limits the expression potential, has been shown to be relaxed in Wilms’s tumours. This paradox could possibly be explained by the operation of a dual mechanism that aims to reduce gene dosage as well as to regulate the production of bioactive IGF-II post-transcriptionally. A clear discrepancy between transcriptional activity and circulating concentrations of IGF-II protein in tumour tissue was in fact discovered more than a decade ago.

The enhancement of cell death by both IGFs in the Wilms’s tumour derived cell line WCCS-1 is the first of its kind to be recorded. However, it remains to be shown whether IGFs can induce cell death on cells from other...
Wilms’s tumours, as well as in other embryonic tumour cells. This work is currently in progress.

This study was supported by grants from Cancerfonden and Barncancerfonden. The support from Professor G Alm and Dr U Blöwe is gratefully acknowledged.