Expression of JNK-interacting Protein JIP-1 and Insulin-like Growth Factor II in Wilms Tumour Cell Lines and Primary Wilms Tumours

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Abstract. *JNK-interacting protein 1 (JIP-1) is an important scaffolding protein in the JNK signalling pathway. It is also believed to play a role in the mediation of mitogenic messages from the plasma membrane to the cell interior. Previous studies suggest that the JIP-gene is co-regulated with the insulin-like growth factor II (IGF II) gene, thereby contributing to the growth stimulatory effects of this potent growth factor. The striking coexpression of these two genes was found in murine fetuses as well as in primary human embryonic tumours. When six primary Wilms tumours were examined, the two genes showed a high degree of co-variation in the sense that high expression of IGF II was followed by high expression of JIP-1 and vice versa. However, when the human Wilms tumour cell line WCCS-1 was examined, a very modest intrinsic expression of IGF II was accompanied by a moderate expression of JIP-1. When exogenous IGF II was added, which has previously been shown to induce apoptosis in this cell line, the JIP-1 expression increased. These data suggest that JIP-1 has a more complex role in the regulation of proliferation as well as programmed cell death.*

Wilms 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 tumour and the early age of bilateral kidney tumour onset suggests that Wilms tumours develop when a predisposing germ-line mutation is accompanied by a second mutation or loss of heterozygosity at the disease locus. (2).

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Fine mapping of the chromosomal locus associated with Wilms 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 (3). One of the first actions revealed for WT1 was its repression of the insulin-like growth factor II (*IGF II*) gene. Wilms tumours are in general characterised by a high rate of expression of the *IGF II* gene (4). IGF II is known to be an ubiquitous and essential growth factor during embryogenesis and acts as a survival factor in embryonic cell lines (5-7). Taken together, this suggests a growth regulatory loop whereby IGF II supports proliferation and/or survival and WT1 counteracts this by specifically suppressing *IGF II* transcription.

However, the general growth stimulatory role of IGFs has been challenged since in specific situations the response to IGF exposure is an increased rate of cell death (8). Even though growth inhibitory effects have been recorded for IGF II and it can act as a tumour suppressor *in vivo*, it was unequivocally shown to be an inhibitor of apoptosis in Wilms tumour cells (9). The rate of IGF II expression has also been shown to be closely correlated to that of a scaffold protein, c-jun NH_2 kinase-interacting protein 1 (JIP-1) (10, 11) believed to be involved in the induction of apoptosis. In this study we investigated Wilms tumour cell response to IGF II and the transcription of the *JIP-1* gene.

Materials and Methods

Primary material and cell lines. Six human Wilms tumours were surgically removed and used for this study. Parts of the tumour were taken for histological classification by light microscopy after staining in haematoxylin/eosin and/or Giemsa. In this study, three human Wilms tumour cell lines were used; WCCS-1 (a kind gift from the late Professor Peter Ekblom), GOS-4 and GaWa (both kind gifts from Professor Christopher F. Graham).

Cell culture. All cell lines were maintained and flasks and wells for experimental purpose were essentially prepared as previously described (12). All tissue culture material was obtained from NUNC (Roskilde, Denmark). The serum-free medium consisted of a 1/1

(v/v) mixture of α MEM lacking nucleosides and deoxynucleosides and Hams F12 medium supplemented with 10 μg/ml of human transferrrin preloaded with iron, in accordance with the manufacturer's instructions. This medium is subsequently referred to as α:Ham.

Two days before a growth experiment, the medium over the stock cultures was replaced by α:Ham supplemented with 10% heat inactivated foetal 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 and counted in a Coulter counter (Coulter Electronics, Bedford, UK). In the subsequent cell culture experiments, the media were pre-equilibrated for at least six hours in an atmosphere of 5% (v/v) CO_2 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 1.4×10^5 and 3.0×10^5 cells per 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 experimental series. At the start of each experiment, cells were rinsed twice in PBS and 10 ml of α:Ham were added to each dish. After one hour, the dishes were briefly removed from the incubator and the IGF II (cf below) 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 in PBS for up to 30 minutes to ensure complete detachment from the cell surface. When all cells had detached, any remaining trypsin was neutralised by adding soybean trypsin inhibitor. The cells were then counted in triplicate in a Coulter counter. The range of the cells of the duplicate rarely exceeded 8% of the mean cell count.

Growth factors. Recombinant IGF-I and IGF II were purchased from British Biotechnology (Oxford, UK). Lyophilised samples were made up to stocks of 2 mg/ml by adding 0.5 ml of 0.1 M acetic acid to 10 mg, shaking every 5 minutes in a 37˚C water-bath and 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 further use. In the growth experiments, The IGF containing vials were thawed on ice, and made up to intermediate stock solutions by diluting with α:Ham to a final concentration of 200 μg IGF II/ml medium. 0.5 ml of this intermediate stock solution was added to each 60 mm dish containing 10 ml serum-free α:Ham. Dishes used for experimental purpose wrer harvested after 24 hours. In each case, serum-free α:Ham and α:Ham supplemented with 10% serum were used as controls.

RNA extraction and Northern blotting. Total RNA from the surgically removed tumours as well as from cultured cells was extracted by a standard Trizol/chloroform extraction procedure (13). In each case, the quality was checked by running the samples on an ethidium bromide containing minigel. Moreover, in each case quantification was carried out by spectrophotometry. From these total RNA samples, polyadenylated RNA was purified using an oligo dT cellulose based purification technique described in (13). One μg of polyadenylated RNA from each tumour was run on a denaturing agarose/formaldehyde gel (13). The electrophoresed RNA was then transferred by blotting onto Hybond N+ filter (Amersham, UK) crosslinked by UV light and stored in a sealed plastic bag until further use.

cDNA probes, radioactive labelling and hybridisation. For the analysis of gene expression, two probes were used: a mouse *IGF II* coding sequence cDNA (from Dr A Shokrai, Uppsala, Sweden) and a 2832 bp murine *JIP-1* cDNA fragment (from Dr A Ward, Bath, UK). The cDNAs were labelled with ^{32}P-dCPT by using a Megaprime DNA labelling system (Amersham UK). The filters were hybridised in a prefabricated hybridisation buffer supplied by Amersham UK, as described in the manufacturer's instructions. After hybridisation, the filters were washed to a stringency level of 0.1xSSC, 55˚C, air-dried and subjected to autoradiography. To obtain a comparable value of the relative *JIP-1* and *IGF II* expression, each film was subjected to densitometry and the integrated *IGF II* and *JIP-1* values were divided by each other. All values were normalised by multiplication with a constant so that the wild-type relationship in a reference embryonic tissue was given the relative value 1 (14).

Results

Figure 1 shows the expression of *IGF II* and *JIP-1* in surgical samples from six histologically confirmed Wilms tumours. From each of the tumours, poly A+ RNA was purified and separated on a gel. The amount of poly A+ RNA loaded onto the gel was quantified by spectrophotometry and checked by running a series of dilutions on a minigel prior to the experiment. To make a valid comparison of the expression pattern of the two genes in the different tumour samples, each filter was first hybridised with *IGF II* cDNA and then stripped of bound probe and rehybridised with *JIP-1* cDNA. Northern blots are shown in Figure 1 upper panel (*IGF II*) and lower panel (*JIP-1*). It was found that the expression levels of both genes differed considerably between the six tumours. However visual examination suggested that there might be a certain amount of co-variation between the expression levels of the two genes. To obtain a more accurate comparison, the intensity of relevant bands was examined after both hybridisations by densitometry and the integrated absorption values divided by each other. All figures were normalised by multiplication with a constant so that the relationship in a reference murine embryo (10) was given the relative value of 1 (14). Table I shows that in all six tumours, the relative values were in the range of 0.7-1.2 which indicates a closely controlled relationship between the transcriptional patterns of the two genes.

Figure 2 shows the expression of *IGF II* and *JIP-1* genes in three Wilms tumour cell lines as determined by Northern blotting. The expression pattern in two of these lines, GaWa and GOS-4, bears a close resemblance to that of the primary

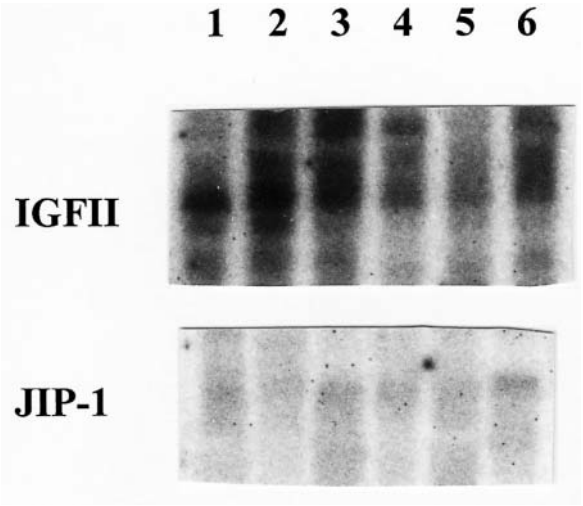

Figure 1. *The expression of IGF II (upper panel) and JIP-1 (lower panel) in six primary Wilms tumours.*

Table I. *The relative expression of the IGF II and JIP-1 genes (arbitrary values). The relationship between IGF II and JIP-1 expression was calculated as a quota based on densitometric measurements of X-ray autoradiographs.*

Tumour no.	*IGFII /JIP-1*
1	0.9
2	1.2
3	1.0
4	0.9
5	0.8
6	1.0

Wilms tumours. Densitometric analysis shows that the relative *IGF II/JIP-1* values were 0.89 and 1.1 respectively. However the third Wilms tumour cell line differed in that the level of *IGF II* transcript was substantially lower than in any primary Wilms tumour or in the other Wilms tumour cell lines. In contrast, the *JIP-1* transcript levels were comparable to those in GaWa and GOS-4.

WCCS-1 cells are unique in the sense that it has been shown that addition of exogenous IGFs triggers apoptosis. Figure 3 shows the expression pattern of *IGF II* and *JIP-1* genes before and 24 hours after addition of 10 μg IGF II/ml medium. It is clearly shown that that the intrinsic expression of the *IGF II* gene is not altered, whereas the transcriptional level of the *JIP-1* gene increases many fold. This is also reflected in the densitometric analysis where the relative *IGF II/JIP-1* value decreased from 0.21 to 0.04 *i.e.* by more than a factor of five.

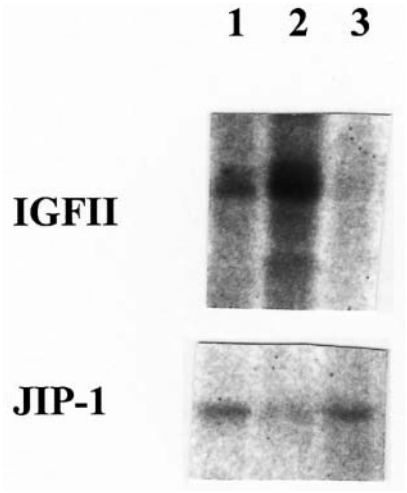

Figure 2. *The expression of IGF II (upper panel) and JIP-1 (lower panel) in three Wilms tumour cell lines: lane 1 GaWa, 2 GOS-4 and 3 WCCS-1.*

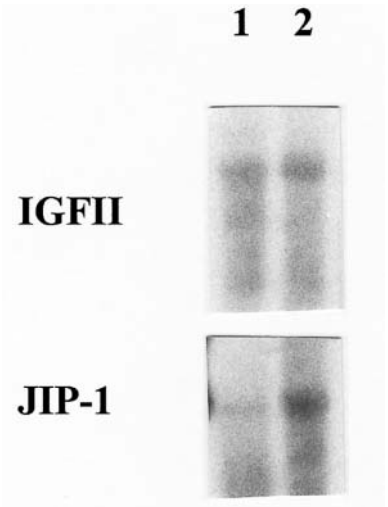

Figure 3. *The expression of IGF II (upper panel) and JIP-1 (lower panel) in WCCS-1 cells before and after 24 h exposure to exogenous IGF II in the culture medium.*

Discussion

The contribution of scaffold proteins to the mitogen-activated protein kinase (MAPK) cascade has been crucial for our understanding of how cell signalling and cellular proliferation are controlled. The initial finding, that a protein, Ste5, was closely involved in the Fus3-MAPK pathway in budding yeast provided the first clue to how protein scaffolds operate (15, 16). Even though it was hard to identify mammalian homologues of Ste5, several scaffold proteins have since been identified (17).

In theory, scaffold proteins play two partly related functional roles. Firstly, they are tools by which cells can

maintain a high degree of specificity in signalling pathways. This is mainly achieved through the co-localisation of molecules that participate in the same signalling pathway to the same area of the cell. In other words, the scaffold proteins increase the efficacy of the pathway as well as ensuring a high degree of specificity. Secondly, scaffold proteins may act as catalysts and thereby activate the different components in the signalling pathway. Ste5, along with MP1 (MAPK partner 1), JIP-1, JSAP-1 (JNK/SAPK-activating protein 1) and KSR (kinase suppressor of Ras) were the first regulatory scaffold proteins with a catalytic role assigned to them (18).

Although the anchoring and catalytic roles of scaffold proteins are judged as combined entities in the literature, it is reasonable to believe that while they act in concert, they may well also act indirectly in their anchoring role. This has, for example, been observed in non-MAPK linked transduction of intracellular signals where the scaffold assembles proteins that are parts of the same signalling chain to a unit, but where the individual molecules do not directly act on each other (reviewed in 19). Nine distinct MAPKs have been described in mammalian cells, namely ERK1/2, ERK3, ERK4, ERK5, ERK6/p38MAPKg, ERK7, ERK8, JNK1/2/3 and p38MAPKa/b/d (20-23). Of these, three groups have been studied in particular detail namely ERK1/2, JNK1/2/3 and p38MAPKa/b/d (reviewed in 24).

Although there is no immediate reason to believe that there is co-regulation of growth factor and scaffold protein gene expression, some data on this matter have been published recently. In the paper reporting the discovery of the first JNK-interacting protein (JIP-1), it was shown that *in vivo* expression in adult mice differed grossly between organs (25). We subsequently showed that these differences were less evident in the mouse embryo (10). These differences in expression pattern pointed at there being some role for JIP-1 in the control of growth and development. IGF II is among the most pivotal growth factors in the mammalian embryo (7, 8, 26, 27). By examining *JIP-1* gene expression in transgenic mice that were heterozygous for a functional *IGF II* gene, it was possible to show that abrogation of IGF II expression was followed by decreased expression of *JIP-1* (10). Moreover, when the expression patterns of *JIP-1* and *IGF II* were examined in primary tumours of embryonic origin (germ cell tumours and Wilms tumours), it was found that irrespective of histological type, the two genes showed a high degree of co-variation in the sense that high *IGF II* expression was followed by high expression of *JIP-1* (11).

A slightly different pattern emerges in this study when a Wilms' tumour cell line came under study. It had previously been reported that IGFs can induce apoptosis in the Wilms' tumour cell line WCCS-1 (9). While this cell line had very low levels of intrinsic *IGF II* expression, it was found to display a level of *JIP-1* expression that was comparable to that of the normal kidney. When exposed to physiological concentrations of IGF II, WCCS-1 cells undergo apoptosis, and also significantly increase their *JIP-1* expression.

This suggests that the increase in *JIP-1* expression in Wilms tumour cells may in some way be linked to the onset of apoptosis. It may also help explain why growth factor addition in this case acts to induce programmed cell death. All MAPK pathways have been implicated in the regulation of apoptosis. More than a decade ago it was shown that the withdrawal of nerve growth factor (NGF) from PC12 cells resulted in apoptosis and that this effect was mediated *via* activation of JNK (28). However, a number of studies have since shown that MAPK pathways can either stimulate or inhibit apoptosis (reviewed in 29). In *MKK-4* knock out mice, it seems clear that JNK plays a protective role since these mice display an increased rate of liver cell apoptosis (30). The survival signals ascribable to JNK are probably mediated by JunD which in turn enhances the transcriptional activity of numerous survival genes. In other systems, JNK has been implicated as a proapoptotic factor. JNK has been shown to phosphorylate the proapoptotic BH3 domain proteins Bim and Bmf that are normally sequestered by binding to myosin and dynein (31). The JNK induced phosphorylation releases these two proteins, thereby facilitating Bax-dependent apoptosis in mitochondria (31). Moreover, JNK can phosphorylate and activate BAD, thereby coupling a stress activated signalling pathway to apoptosis (32). Another proposed role for JNK in promoting apoptosis is by inactivating protective proteins. Such targets include Bcl-X and Bcl-2 (33, 34) and it appears that this process depends upon Bax (35).

Interleukin 1-beta (IL-1β) acts as a pro-apoptotic stimulus in pancreatic beta cells (36). However this activation process is parallelled by a decrease in JIP-1b expression (36). Conversely, overexpression of JIP-1b significantly inhibits IL-1β-induced apoptosis. Presumably, pancreatic beta cells may constitute a special case where the variations in JIP-1b expression regulate the susceptibility to cytokine-induced apoptosis irrespective of JNK signalling (37). More recently, another JNK signalling scaffold protein, POSH, was found to link active, GTP-bound Rac1 to downstream JNK in an apoptotic pathway (38). Moreover POSH interacts directly with JIP-1 and Kukesov *et al*. (39) were able to demonstrate that the POSH-JIP-1 complex is specifically involved in the apoptosis-inducing process. Both POSH and JIP-1 can independently interact with members of all three MAPK tiers. However, the direct link between POSH, JIP-1 and JNK provides us with an interesting possibility to explain how scaffold proteins contribute to the apoptotic pathway.

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