The Expression of the Insulin-like Growth Factor II, JIP-1 and WT1 Genes in Porcine Nephroblastoma

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Abstract. *c-Jun N-terminal kinase (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 have suggested 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 co-expression of these two genes has been found in murine foetuses as well as in primary human embryonic tumours. When six primary Wilms tumours (nephroblastomas) from pig were examined, the two genes showed a high degree of co-variation in the sense that high or low expression of IGF-II and high or low expression of JIP-1 ocurred together. By contrast the expression of a third Wilms tumour-related gene, WT1, was completely uncorrelated to the expression of IGF-II and JIP. In this respect, porcine nephroblastomas resemble human Wilms tumours. This further suggests that JIP-1 may play a role in the regulation of IGF-II-driven tumour cell proliferation.*

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). In humans, it is one of the most common pediatric malignancies affecting about 1 in 100,000 children, usually during the first five years of life. 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 germline mutation is accompanied by a second mutation or loss of heterozygosity at the disease locus (2). Fine mapping of the chromosomal locus associated with Wilms tumour has led to the cloning and characterisation of the Wilms tumour related 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 data suggests a growth regulatory loop whereby IGF-II supports proliferation and/or survival and WT1 counteracts this by specifically suppressing IGF-II transcription. Recent evidence has suggested that a third gene encoding c-Jun *N*-terminal kinase (JNK) interacting protein (JIP-1) a scaffold protein involved in the regulation of JNK signalling, is co-regulated with IGF-II in a variety of embryonic tissues and tumour cells (8-10).

Although spontaneous Wilms tumours or nephroblastomas occur in the kidneys of mammals such as cattle, dogs and rabbits, it develops most frequently in swine. Porcine nephroblastomas are histologically very similar to human Wilms tumours, and moreover normally develop at the age of 1, which given the lifespan of a pig corresponds extremely well to the situation in humans (11). There have been attempts to characterise Wilms tumours in pigs on a molecular level, thereby providing a potential model for the development of this enigmatic tumour in humans. Moreover the genes for IGF-II (12, 13) and WT1 (11) have already been cloned and characterized in pigs and the expression at different developmental stages has been studied (11, 13). This study aimed at examining how JIP-1 is expressed in normal pig kidney as well as in porcine nephroblastoma tissue and its relationship with IGF-II and WT1.

Materials and Methods

Primary material. Six porcine nephroblastomas were collected from abbatoirs in Sweden and used for this study. Parts of the tumours were taken for histological classification by light microscopy after staining in haematoxylin/eosin and/or Giemsa. In this study, one

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Key Words: JIP-1, IGF-II, WT1, Wilms tumour.

snap-frozen kidney from a 4-day-old piglet and another from a 2-year-old sow were used as controls

RNA extraction and Northern blotting. The total RNA from samples from the surgically removed tumours was extracted by a standard Trizol/chloroform extraction procedure. 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 serial dilution and spectrophotometry. From these total RNA samples, polyadenylated RNA PolyA+ RNA) was purified using an oligo dT cellulose based purification technique described in (14). One μg of PolyA+ RNA from each tumour was run on a denaturing agarose/formaldehyde gel (14). The electrophoresed RNA was then transferred by blotting onto Hybond N+ filter (Amersham, Little Chalfont, 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, three probes were used: a 2832 bp murine JIP-1 sequence cDNA (from Dr R Davis through Dr A Ward, Bath, UK), a mouse IGF-II coding sequence cDNA (from Dr A Shokrai, Uppsala, Sweden) and a 588 bp human WT1 cDNA fragment (from Dr A Ward, Bath). The cDNAs were labelled with ^{32}P-dCTP (cytidine triphosphate) by using a Megaprime DNA labelling system (Amersham, Little Chalfont, UK). The filters were hybridized in a prefabricated hybridization buffer supplied by Amersham (Littel Chalfont,UK), as described by the manufacturers instructions. After hybridization, the filters were washed to a stringency level of 0.1xSSC (standard sodium citrate), 55˚C, airdried and subjected to autoradiography. To make a valid comparison of the expression pattern of the two genes in the different tumour samples, each filter was first hybridized with IGF-II cDNA and then stripped of bound probe and rehybridized with JIP-1 cDNA, subjected to repeated washes and finally rehybridized with WT1. A parallel filter was checked for the success of the washing after each step of the procedure. 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 the values were normalized by multiplication with a constant so that the wild-type relationship in a reference murine embryonic tissue (10) was given the relative value 1 (15).

Results

Figure 1 shows the expression of IGF-II and JIP-1 in surgical samples from six histologically confirmed Wilms tumours. All six tumours were classified as nephroblastic in which at least two thirds of the tumour consisted of undifferentiated mesenchymal blastemal cells. The expression levels of IGF-II as well as JIP-1 differed only slightly between the six tumours. Moreover visual examination suggested that there might be a certain amount of covariation between the expression levels of the two genes. The Wilms tumours displayed both embryonic IGF-II transcripts of 5.4 and 2.3 kB. JIP-1 was expressed as a single 3.0 kB transcript. Table I shows that in all six tumours, the relative IGF-II/JIP-1 values after normalization with the reference value were in the range of 0.8-1.2, which indicated a closely controlled relationship between the transcriptional patterns of the two genes. In contrast, there was a huge variation in WT1 expression between the six tumours (data not shown). There was no visual correlation between the expression of the WT-1 and IGF-II/JIP-1 genes in these tumours.

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

Tumour no.	IGF-II	JIP-1	IGF-II/JIP-1
1.	+++	+	0.9
2.	+++	+	1.2
3.	+++	+	1.0
4.	+++	+	0.9
5.	+++	+	0.8
6.	+++	+	1.0

Figure 2 shows the expression of IGF-II, JIP-1 and WT1 genes in primary porcine tissues as determined by Northern blotting. The expression pattern in a histologically normal kidney from a 4-day-old piglet as well as that from an adult sow showed very weak IGF-II expression, displaying the 4.7 and 1.2 kB adult transcripts which correspond to the sizes previously shown (13). Furthermore, both tissues expressed a 3 kB JIP-1 transcript at comparable levels. Finally the previously demonstrated difference between WT1 expression in the newborn and adult normal kidney (11) was confirmed. A clear 3 kB band was observed in the newborn tissue but was conspicuously absent in the adult kidney.

Discussion

The six primary porcine nephroblastomas expressed the IGF-II gene at a pronounced level. Even though there was some variation between the indvidual samples, each tumour expressed IGF-II, a high level, far beyond that of the normal embryonic or adult kidney. By contrast, there was a huge variation in the expression of the WT1 gene, normally attributed to the central regulation of Wilms tumour development (1, 11). However, as in line with previous studies (8-10) a close covariation was demonstrated between IGF-II expression and the transcriptional level of the key scaffold protein JIP-1.

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. In theory, scaffold proteins play two partly related functional roles. Firstly, scaffold proteins are tools by which cells can maintain a high degree of specificity in signalling pathways. This is mainly achieved

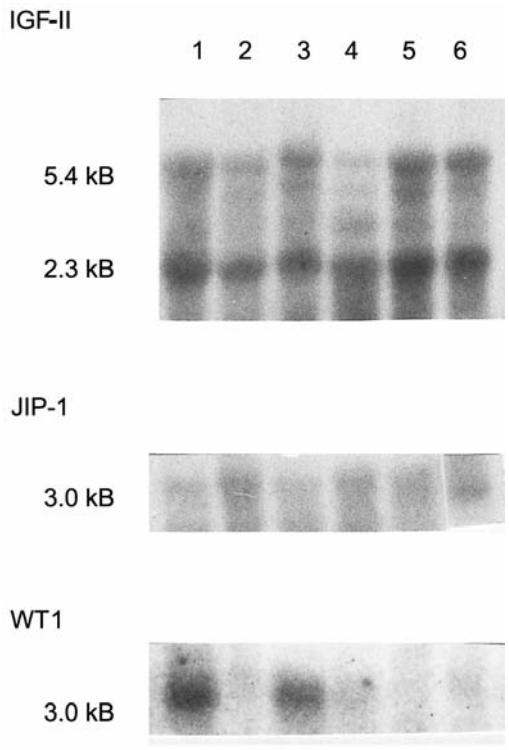

Figure 1. *The expression of IGF-II, JIP-1 and WT1, by Northern blot, in six primary porcine nephroblastomas.*

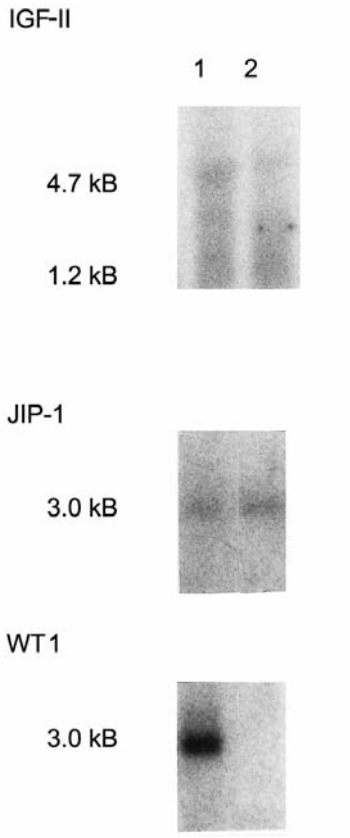

Figure 2. *The expression by Northern blot of IGF-II, JIP-1 and WT1 in a kidney from a 4-day-old piglet (1) and a kidney from a 2-year-old sow (2).*

through the co-localization 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. Although the anchoring and catalytic roles of scaffold proteins are judged as combined entities, 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 16).

Although there is no immediate reason to believe that growth factor and scaffold protein gene expression is co-regulated, 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 (17). We subsequently showed that these differences were less evident in the mouse embryo (8). These differences in expression pattern pointed at 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 (4-7, 19, 20). 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 (8). Moreover, when the expression patterns of JIP-1 and IGF-II were examined in primary tumours of human embryonic origin (germ cell tumours and Wilms tumours), it was found that irrespective of histological type, the two genes showed a high degree of covariation in the sense that high IGF-II expression was followed by high expression of JIP-1 (9, 10). In this respect, porcine nephroblastoma resembles the human Wilms tumour.

It has previously been suggested that an 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 (a phaeochromocytoma cell line) cells resulted in apoptosis and that this effect was

mediated *via* activation of JNK (21). However a number of studies have since shown that MAPK pathways can either stimulate or inhibit apoptosis (reviewed in 22). In MAP kinase kinase 4 (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 (23). 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 (24). JNK induced phosphorylation releases these two proteins, thereby facilitating Bax-dependent apoptosis in mitochondria (25). Moreover, JNK can phosphorylate and activate BAD, thereby coupling a stress activated signalling pathway to apoptosis (26). Another proposed role for JNK in promoting apoptosis is by inactivating protective proteins. Such targets include Bcl-X and Bcl-2 (27, 28) and it appears that this process depends upon Bax (28).

Interleukin 1-β (IL-1β) acts as a pro-apoptotic stimulus in pancreatic beta cells (29). However this activation process is parallelled by a decrease in JIP-1β expression (29). Conversely, overexpression of JIP-1β significantly inhibits IL-1β induced apoptosis. Presumably, pancreatic beta cells may constitute a special case where the variations in JIP-1β expression regulate the susceptibility to cytokine induced apoptosis irrespective of JNK signalling (30).

The data presented in this study suggest that JIP-1 remains an interesting intermediary in the regulation of growth and possibly also in apoptosis in porcine nephroblastoma. The puzzling finding that WT-1 expression varied considerably remains to be further investigated. This work is currently in progress.

Acknowledgements

This study was supported by Cancerfonden, Barncancerfonden and the Cancer Research Campaign of Great Britain. WE was the recipient of a UICC (International Union against Cancer) technology transfer fellowship. The authors acknowledge the gift of the JIP-1 cDNA probe from Dr RJ Davis and the IGF-II cDNA probe from Dr Arman Shokrai

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Received August 12, 2009
Revised November 2, 2009
Accepted November 5, 2009