Bmp, Fgf and Wnt signalling in programmed cell death and chondrogenesis during vertebrate limb development: the role of Dickkopf-1

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ABSTRACT  Dickkopf-1 (Dkk-1) is a potent head inducer in Xenopus. This effect can be attributed to its capability to specifically inhibit Wnt/β-catenin signalling. Recent data point to a crucial role for Dkk-1 in the control of programmed cell death during vertebrate limb development. In this paper, we present a comparative expression analysis of Dkk-1, Bmp-4 and Sox-9 as well as data on the regulation of Dkk-1 by Wnt. Finally, we summarize the current knowledge of its potential function in the developing limb and present a model how the interplay of the Bmp, Fgf and Wnt signalling pathways might differentially regulate programmed cell death versus chondrogenic differentiation in limb mesodermal cells.

KEY WORDS: Bmp, Dkk-1, limb development, programmed cell death, Wnt

Introduction

The vertebrate limb provides a paradigm for developmental programmed cell death (PCD). Morphogenesis of this structure critically depends on the endogenous suicide program eliminating cells in very confined regions within the early limb bud (Hurle et al., 1996). These regions include the so-called anterior necrotic zone (ANZ), its posterior counterpart (PNZ) as well as the interdigital mesenchyme (INZ). Not much is known about the molecules that control PCD in the aforementioned areas. It seems that a complex interplay between different members of the Tgfβ and Fgf families largely contributes to this activity (Merino et al., 1998; Macias et al., 1999; Montero et al., 2001). Members of the Bmp subfamily have been identified as components triggering PCD, especially within the INZ (Yokouchi et al., 1996; Zou and Niswander, 1996). Remarkably, Bmps have originally been identified by their ability to induce bone structures (Wozney et al., 1988). Indeed, these signalling molecules are also crucial for the formation of condensations in the vertebrate limb (Pizette and Niswander, 2000). Thus, in very close vicinity to the regions where Bmps promote PCD, the activity of the very same molecules leads to a completely different reaction of the mesodermal cells. Interestingly, both activities of Bmps seem to be mediated by BmpR-Ib (Zou et al., 1997a). The use of different receptors might thus not be the basis for this dual function, the nature of which remains obscure until today.

Dickkopf-1 (Dkk-1) is a member of a new family of secreted proteins which was isolated in Xenopus (Glinka et al., 1998). Homologues have now been identified in different vertebrate species like chick, fish, mouse and also humans (Glinka et al., 1998; Krupnik et al., 1999; Hashimoto et al., 2000; Shinya et al., 2000; Mukhopadhyay et al., 2001). Dkk-1 represents a potent antagonist of the Wnt/β-catenin signalling pathway (reviewed in Zorn, 2001). We and others have previously described the dynamic expression pattern of Dkk-1 as well as its potential function in modulating PCD during vertebrate limb development (Grotewold et al., 1999; Mukhopadhyay et al., 2001; Grotewold and Rüther, 2002). In this paper, we present a comparative expression analysis of Dkk-1, Bmp-4 and the cartilage-specific Sox-9. We also analyzed Dkk-1 expression and the extent of cell death in different mouse limb mutants and its transcriptional regulation by diverse ligands of the Wnt family. Finally, we summarize the recent advances of our understanding of Dkk-1 function during vertebrate limb development and present a model involving Dkk-1 that might explain how limb mesodermal cells become determined to either undergo chondrogenic differentiation or to be committed to apoptotic cell death in response to Bmp signals.

Abbreviations used in this paper: AER, apical ectodermal ridge; ANZ, anterior necrotic zone; Bmp, bone morphogenetic protein; Dkk-1, Dickkopf-1; ES cell, embryonic stem cell; Fgf, fibroblast growth factor; Ft, Fused toes; Hx, Hemimelic extra toe; INZ, interdigital necrotic zone; PCD, programmed cell death; PNZ, posterior necrotic zone; Tgfβ, transforming growth factor β; Xi J, Extra-toes J.

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Results

Dkk-1 is co-expressed with Bmp-4

At E10.5 Dkk-1 is expressed in an anterior as well as a posterior mesodermal domain in the mouse limb bud (Fig. 1A). Later on, these domains become more confined and transcripts can additionally be detected in the apical ectodermal ridge (AER, Fig. 1C) before expression in the interdigital mesenchyme starts (Fig. 1E). As this pattern of expression seemed to be quite similar to that of some members of the Bmp family we undertook a comparative analysis of Bmp-4 transcription at the corresponding time points. Indeed, at all developmental stages examined (E10.5–E12.5) the expression domains of Bmp-4 and Dkk-1 overlapped to a high degree (compare Fig. 1 A,B; C,D; E,F).

Dkk-1 Expression is associated with the Sites of PCD

We have previously shown that the areas of Dkk-1 expression correspond to the sites of PCD in both, mouse and chick limb development (Grotewold and Rüther, 2002). Interestingly, Dkk-1 is ectopically expressed in limb buds of polydactylosus XtJ/XtJ mutant embryos while it is normally transcribed in limb buds of Hx+/+ embryos (Grotewold and Rüther, 2002) which also develop ectopic preaxial digits. We asked whether the enhanced activity of Dkk-1 might affect PCD in the anterior mesoderm of XtJ/XtJ limb buds. The TUNEL stainings in Fig. 2 show that while the extent of cell death is slightly reduced in the anterior mesoderm of Hx+/+ limb buds (Fig. 2B) compared to the wild-type (Fig. 2A), it is clearly increased in the XtJ/XtJ limbs (Fig. 2C). The increased PCD might not affect digit number in the polydactylosus XtJ/XtJ embryos but rather digit length as the ectopic digits are significantly shorter than the preaxial extra digit of Hx+/+ embryos (data not shown). Thus, in normal as well as mutant limb development, the sites of Dkk-1 expression are associated with high PCD.

Dkk-1 Expression is excluded from Regions of Chondrogenesis

To analyze the relationship between the regions of Dkk-1 expression and sites of PCD and on the other hand the areas of chondrogenesis in more detail, we carried out a comparative analysis of Dkk-1 and Sox-9 expression which marks the cartilagenous skeleton (Bi et al., 2001). At E12.5 transcription of the two genes is clearly mutually exclusive with Dkk-1 being expressed in the INZ and Sox-9 within the digital rays (Fig. 3 A,C). This pattern is maintained one day later in development (Fig. 3 E,G). In order to ask whether this complementary expression is also realized during mutant limb development, we analyzed expression of the two genes in limb buds of Ft+/+ embryos. These mice develop a syndactyly due to ectopic bone elements connecting digits 1-4 (Heymer and Rüther, 1999). At E12.5 Dkk-1 is ectopically expressed in the anterior/distal part of Ft+/+ limb buds (Fig. 3B). We have previously shown that PCD is also enhanced in the corresponding region (Grotewold and Rüther, 2002). In parallel, Sox-9 starts to be misexpressed in the anterior/distal part of Ft+/+ limb buds. The domain of ectopic Sox-9 transcripts, however, does not seem to overlap with that of ectopic Dkk-1 expression but to border proximally on this domain (compare Fig. 3 B,D). Thus, the region of ectopic bone formation is separated from the area of enhanced Dkk-1 expression and PCD. At E13.5 Dkk-1 transcripts are lost in the region of PCD.
from the distal-most part of Fl/+ limb buds but are restricted to the interdigital regions (Fig. 3F). At this time point, the ectopic Sox-9 expression domain has expanded to cover the complete region of the presumptive fusion of digits 1-4 (Fig. 3H). Thus, also in Fl/+ mutant limbs Dkk-1 expression is excluded from the regions of chondrogenesis and the dynamics of its transcription correlates with the temporary alterations of PCD.

**Regulation of Dkk-1 by Wnt**

It seems to be a recurrent theme in animal development that secreted signalling molecules induce the expression of inhibitors of their own activity, probably to limit the range of their action. This is e.g. true for the Bmp-inhibitor Bambi (Onichtchouk *et al.*, 1999, Grotewold *et al.*, 2001) and the Fgf-antagonizing Sproutys (Minowada *et al.*, 1999). We wanted to analyze whether Dkk-1 which inhibits Wnt/β-catenin signals might be transcriptionally induced by members of the Wnt family. Arnold *et al.* (2000) and Lickert *et al.* (2000) recently reported a co-culture system of embryonic stem (ES) cells with NIH/3T3 fibroblasts that express different Wnt genes. The induction of Wnt target genes like e.g. Cdx-1 could then be observed in the ES cells (Lickert *et al.*, 2000). Using this system we could reproduce the induction of Cdx-1 by Wnt-1, -3a and -4 and slightly also by Wnt-7a and -7b (Fig. 4), verifying that this system worked in our hands. When we monitored Dkk-1 transcription by RT-PCR we observed a transcriptional induction of the gene by Wnt-1, -3a and -4 (Fig. 4). Thus, Dkk-1 expression responds to the activity of a subset of Wnt ligands.

**Discussion**

Until recently, not much was known about potential functions of Dkk-1 outside head induction (Glinka *et al.*, 1998). We and others could show that the spatiotemporal expression of the gene during vertebrate limb development coincides significantly with the sites of PCD (Grotewold *et al.*, 1999; Mukhopadhyay *et al.*, 2001; Grotewold and Rüther, 2002). This is not only true for normal but also mutant mouse limb development as shown for syndactylyous Fl/+ mutant embryos and the polydactylous Xt/Xt' embryos (Grotewold and Rüther, 2002 and this study). Moreover, the co-expression of Dkk-1 with some members of the Bmp family and their target genes suggested a role for Dkk-1 in the Bmp-triggered PCD cascade. Indeed, Dkk-1 is transcriptionally regulated by Bmp (Mukhopadhyay *et al.*, 2001; Grotewold and Rüther, 2002). Remarkably, Dkk-1 is only induced by Bmp under PCD-inducing conditions but not when the Bmp signal promotes the formation of bone. Moreover, Bmp activity is also necessary for the endogenous expression of Dkk-1 (Grotewold and Rüther, 2002).

Dkk-1 is also positively regulated by and most likely dependent on the activity of Fgf signals from the AER (Grotewold and Rüther, 2002). We now show that also Wnt-1, Wnt-3a and Wnt-4 induce the expression of Dkk-1. Wnt-1 and -3a activate an intracellular signaltransduction pathway which leads to the stabilization of β-catenin (Minowada *et al.*, 1997). Evidence has also been reported for Wnt-4 to signal via β-catenin in chick limb development (Hartmann...
and Tabin, 2000). As Dkk-1 specifically interferes with the Wnt/β-catenin pathway (Zorn, 2001), it thus seems, that these ligands limit their range of action by the induction of Dkk-1.

During chick limb development, Fgf-10 induces the expression of Wnt-3a in the AER (Kawakami et al., 2001). The induction of Dkk-1 by Fgf might thus be mediated by Wnt ligands, in particular Wnt-3a, which is a crucial component for the correct establishment of the AER (Kengaku et al., 1998; Kawakami et al., 2001). Besides, it has been shown in Xenopus that Dkk-1 can inhibit the activity of Wnt-3a (Kazanskaya et al., 2000). These interactions might explain the consequences of Dkk-1 overexpression during chick limb development. Ectopic expression of Dkk-1 with an adenooviral and a retroviral system led to dramatic distal truncations of the resulting limb buds (Mukhopadhyay et al., 2001; Grotewold and Rüther, 2002). The truncated limbs exhibited massive PCD (Grotewold and Rüther, 2002). This phenomenon, however, seemed to appear secondary to the truncations as we could not detect a significant increase in the number of apoptotic cells until truncations were rather advanced. Thus, we conclude that Dkk-1 blocks the regulatory loop between Fgfs and Wnt-3a which ensures AER maintenance and thus interferes with distal outgrowth. This interpretation is supported by the expansion of the Fgf-8 expression domain and most likely the AER itself in Dkk-1-/- limb buds (Mukhopadhyay et al., 2001).

As mentioned above, overexpression of Dkk-1 in the limb bud does not seem to directly commit cells towards a death program which is consistent with findings in cultured cells (Wang et al., 2000). Nevertheless, two lines of evidence support a crucial role of Dkk-1 in PCD. First, the ablation of Dkk-1 function in the mouse led to syndactyly and the variable appearance of ectopic anterior and posterior digits, a phenotype that strongly suggests reduced PCD in the ANZ, PNZ and INZ, respectively, to be the basis for these alterations (Mukhopadhyay et al., 2001). Second, we could recently show that overexpression of Dkk-1 does have a dramatic influence on Bmp-triggered PCD. When a bead soaked in Bmp protein is implanted into the undifferentiated mesenchyme of early limb buds, PCD is induced in a restricted area around the bead after several hours (Macias et al., 1997; Zou et al., 1997b; Pizette and Niswander, 1999). When limb buds were infected with a retrovirus expressing Dkk-1 before the bead implantation this led to a dramatic increase in the amount of cells undergoing PCD (Grotewold and Rüther, 2002). Thus, it seems that the prior exposure of a cell to Dkk-1 significantly enhances the ability of Bmp to induce PCD. One could imagine that the Dkk-1-mediated inhibition of Wnt/β-catenin signalling might provide a permissive signal that confers mesodermal limb bud cells the competence to react on an instructive Bmp signal to initiate the endogenous death program. We thus believe that the status of Wnt signalling of a mesodermal cell determines whether the cell undergoes PCD or chondrogenic differentiation in response to a Bmp signal. Thus, under conditions where both, Bmp and Wnt/β-catenin signalling exceed a certain level of activity this would lead to chondrogenesis to occur (Fig. 5). Indeed, enhanced Wnt/β-catenin signalling is associated with accelerated chondrogenesis in chick limb development (Hartmann and Tabin, 2000).

Further experimental proof will be required to evaluate whether our model might hold true, but up to now it certainly provides an attractive possibility to explain the dual role of Bmp signalling in vertebrate limb development.

Materials and Methods

In Situ Hybridization and TUNEL Staining

Whole mount in situ hybridization has been carried out according to standard procedures (Xu and Wilkinson, 1998) using the following probes: Bmp-4 (kindly provided by Dr. B. Hogan), Dkk-1 (Glinka et al., 1998), Sox-9 (kindly provided by Dr. R. Lovell-Badge). Whole mount TUNEL was performed as described (Grotewold and Rüther, 2002).

Co-Culture of NIH/3T3 and ES Cells

For co-culture experiments we used NIH/3T3 fibroblasts which were stably transfected with expression constructs of the following genes: lacZ, Wnt-1, Wnt-3a, Wnt-4, Wnt-5a, Wnt-7a and Wnt-7b (kind gifts from Dr. R. Kemler) which were plated at 10^5 cells /10 cm dish one day before addition of the ES cells. 2-3 days before co-culture, ES cells (ES 14-1, Kühn et al., 1991) were transferred to gelatine-coated dishes to remove the feeder cells. 2x10^6 ES cells were added to the NIH/3T3 cells and incubated for 24h. Cells were washed with PBS and RNA was isolated using TRIZOL (GibcoBRL). First strand synthesis was performed using the Expand RT system (Roche) according to the manufacturer’s instructions.

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References


Dkk-1 in Limb Development


