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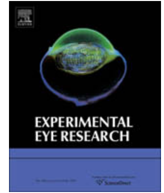

TGFβ promotes Wnt expression during cataract development

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A B S T R A C T

TGFβ induces lens epithelial cells to undergo epithelial mesenchymal transition (EMT) and many changes with characteristics of fibrosis including posterior capsular opacification (PCO). Consequently much effort is directed at trying to block the damaging effects of TGFβ in the lens. To do this effectively it is important to know the key signaling pathways regulated by TGFβ that lead to EMT and PCO. Given that Wnt signaling is involved in TGFβ-induced EMT in other systems, this study set out to determine if Wnt signaling has a role in regulating this process in the lens. Using RT-PCR, in situ hybridization and immunolocalization this study clearly shows that Wnts 5a, 5b, 7b, 8a, 8b and their Frizzled receptors are upregulated in association with TGFβ-induced EMT and cataract development. Both rat in vitro and mouse in vivo cataract models show similar profiles for the Wnt and Frizzled mRNAs and proteins that were assessed. Currently it is not clear if the canonical β-catenin/TCF signaling pathway, or a non-canonical pathway, is activated in this context. Overall, the results from the current study indicate that Wnt signaling is involved in TGFβ-induced EMT and development of fibrotic plaques in the lens.

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1. Introduction

Approximately 25 million people are bilaterally blind as a result of cataract, which is the most common cause of blindness in the world today (Chang et al., 2007). The most effective treatment currently for cataract is surgery, which involves removal of opaque lens material and insertion of a plastic intraocular lens into the capsular bag. Although initially very effective in restoring sight, a common postoperative complication of surgery is the development of a secondary cataract commonly known as posterior capsule opacification (PCO). This is caused by fibrotic growth of residual lens epithelial cells left behind in the capsular bag after surgery (Wormstone, 2002). Studies with rat and mouse models showed that TGFβ induces lens fibrosis and cataract. TGFβ induces rodent lens epithelial cells to undergo epithelial mesenchymal transition (EMT) and acquire many features and markers characteristic of fibrosis including PCO (Hales et al., 1994, 1995; Liu et al., 1994; Lovicu and McAvoy, 2005; Lovicu et al., 2002, 2004a,b). Studies on human lens cells in vitro, as well as analysis of postoperative cataract material, have also implicated TGFβ as a key inducer of fibrotic cataracts including PCO (Wormstone, 2002; Saika, 2004).

As PCO progresses, further treatment such as Nd–YAG laser capsulotomy is often given to try to restore some visual acuity; however, it is not without its complications and adds significant costs to what is the most common surgery carried out in Western countries (Saika, 2004; Billotte and Berdeaux, 2004). Therefore, a major focus in cataract research in recent years has been directed at identifying ways of blocking the cataractous effects of TGFβ and promoting the normal lens epithelial phenotype (Wormstone et al., 2002; Saika et al., 2001; Schulz et al., 1996; Stump et al., 2006). However, to do this effectively it is important to know the key signaling pathway(s) regulated by TGFβ that leads to EMT and all the PCO-associated changes. This is central to devising molecular strategies to slow or prevent this common complication of modern cataract surgery.

TGFβ is a key regulator of many processes in both normal and pathological development (Nawshad et al., 2005). On TGFβ receptor activation, receptor Smads (Smad2 or Smad3) associate with Smad4. This Smad2/3–Smad4 complex then enters the nucleus to regulate transcription of target genes. Amongst the major effects of TGFβ signaling is induction of EMT and associated fibrosis (Akhurst and Derynck, 2001). Studies have also shown that Smad2 and Smad3 have non-redundant functions and that Smad3 appears to be the key Smad regulator of TGFβ-induced EMT/fibrosis (Yang et al., 2003; Roberts et al., 2006).

Studies have also highlighted the importance of Smad-independent TGFβ-activated pathways (Derynck and Zhang, 2003). With particular importance for EMT, TGFβ has been shown to activate mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and Rho GTPases (Peinado et al., 2003; Yi et al., 2005).

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In addition, and in the context of mediating the cellular processes of EMT, TGFβ has been shown to stimulate the β-catenin/TCF pathway (Nawshad et al., 2005; Medici et al., 2006; Warner et al., 2005). β-Catenin/TCF signaling is involved in regulating EMT in development and cancer (Brabletz et al., 2005; Reya and Clevers, 2005). β-Catenin is tightly regulated by a multiprotein complex containing glycogen synthase kinase-3β (GSK3β). This complex phosphorylates β-catenin and targets it for degradation. Signaling events that activate Disheveled (Dvl) phosphorylate GSK3β and inhibit this cytostolic degradation machinery and promote stabilization of β-catenin. In this unphosphorylated form β-catenin can translocate to the nucleus and associate with the DNA binding factors, TCF or LEF, and regulate expression of target genes that influence many different cellular processes. Through binding to Frizzled receptors, members of the Wnt growth factor family are well known activators of Dvl and subsequently this, so called, canonical signaling pathway (Reya and Clevers, 2005). In relation to mechanisms of cross-talk between Wnt and TGFβ pathways, Smad3 has been shown to bind Dvl (Warner et al., 2003) and TGFβ/Smad activated ILK, phosphorylates (inactivates) GSK3β and can lead to stabilization of β-catenin (Willis and Borok, 2007).

Studies on the lens, using in vitro and in vivo models, have shown that exposure to TGFβ and subsequent EMT and fibrotic changes are associated with translocation of Smad3 into the nucleus. This has been shown in the development of anterior subcapsular cataracts (involves EMT and fibrosis similar to PCO) that arise in mouse lenses cultured with TGFβ as well as in a mouse model where a wound healing response is induced by injury (Saika et al., 2001, 2004). In addition, studies with human lens cells have shown that nuclear translocation of Smad3 is associated with the EMT and the PCO that follows cataract surgery (Saika et al., 2002, 2004). However, recent studies have also shown that when a Smad3 knockout mouse is crossed with a transgenic mouse that overexpresses TGFβ1 in lens cells, they still undergo a strong EMT and fibrotic response (Banh et al., 2006). This result indicates that Smad-independent signaling pathways may also have major roles in mediating TGFβ-induced EMT and fibrosis in the lens.

As already noted for other systems, TGFβ-induced EMT has been shown to be associated with β-catenin/TCF signaling and this is characterized by nuclear translocation of unphosphorylated (stabilized) β-catenin (Medici et al., 2006; Jian et al., 2006). Given this background, it is important to determine if TGFβ-activation of β-catenin/TCF signaling has a role in regulating EMT/fibrosis in the lens. In a first step to address this question, this study set out to investigate the effects of TGFβ on Wnt expression and signaling in rat lens explants and transgenic mice.

2. Materials and methods

All procedures involving animals were carried out in accordance with the National Health and Medical Research Council (Australia) guidelines and conformed to the Association for Research in Vision and Ophthalmology Incorporated Resolution on the use of animals in Ophthalmic Research. All procedures involving animal and human tissues were approved by the Animal and Human Ethics Committees of the University of Sydney, Australia.

2.1. Tissue collection and processing

For transgenic studies, eyes were collected from transgenic mice (OVE853) that overexpress an active form of TGFβ1 specifically in the lens (see Lovicu et al., 2002, 2004a,b; Srinivasan et al., 1998). Tissues were collected from postnatal day (P3) to P21 transgenic and wild-type mice. All tissues collected were fixed in 10% neutral buffered formalin (NBF) overnight, rinsed in PBS and processed for histology. At least two litters each of transgenic and wild-type mice were collected and from these at least 3 pairs of eyes were sectioned for analysis.

For tissue culture studies, Wistar rats between P19 and P25 were sacrificed by asphyxiation and their eyes removed. Whole lenses were dissected and cultured for 5 days in medium 199 supplemented with 1 ng/ml TGFβ2 (R&D Systems, Inc., Minneapolis, MN) as described earlier (Hales et al., 1995). Lens epithelial explants (a monolayer of lens epithelial cells attached to their natural basement membrane, the lens capsule) were prepared as described previously (Lovicu and McAvoy, 2001) and cultured with or without TGFβ2 for 3 days. For explants, TGFβ2 was used at a final concentration of 200 pg/ml as earlier studies showed this dose to be potent at inducing prominent cataractous changes in explants by 3 days of culture (Gordon-Thomson et al., 1998). For both whole lens and explant experiments culture dishes, each containing 2 lenses or explants, were cultured with or without TGFβ2 (controls). At least 5 dishes were in each group and experiments were repeated at least 3 times.

2.2. RT-PCR

RT-PCR was carried out on P19–25 rat lenses cultured with TGFβ2 for 5 days. Total RNA was extracted from dissected lens capsule (with adherent epithelial cells) using Tri Reagent (Sigma, Sydney Australia). First-strand cDNA synthesis was carried out using 2 μg of RNA with a reverse transcription system (Promega, Sydney, Australia) according to the manufacturer's instructions. RT-PCR for Wnts 5a, 5b, 7a, 7b, 8a and 8b was carried out as described previously (Stump et al., 2003).

2.3. In situ hybridization

The expression patterns of mRNA transcripts for Wnts 5a, 5b, 7a, 7b, 8a, 8b and Frizzled 2 in rat lenses and in lenses of transgenic and wild-type mice were examined by in situ hybridization using digoxigenin-labeled riboprobes (Stump et al., 2003). Sense and anti-sense probes were prepared as described previously (Stump et al., 2003). The in situ hybridization procedures were conducted as previously described for digoxigenin-labeled riboprobes (de Iongh et al., 2001).

2.4. Immunofluorescence

For both transgenic and cultured lenses, 6 μm mid-sagittal sections were used for immunolocalization of Wnts 5a, 7b and Frizzleds (antibodies from Santa Cruz Biotechnology, CA; for Frizzled, the H-300/sc-9196 antibody was used as it recognizes Frizzled proteins 1–10). A monoclonal antibody (Dako, Glostrup, Denmark) was used to localize α-smooth muscle actin. Activated (unphosphorylated) β-catenin was localized in lens epithelial explants with an antibody to the unphosphorylated form (Clone 8E7, Upstate, Lake Placid, NY). For immunofluorescent labeling, explants were fixed in methanol. Following standard blocking and washing procedures explants or whole lenses were incubated overnight at 4 °C with the primary antibody then incubated with an appropriate secondary antibody (Alexa 488 or Alexa 594; Invitrogen, Mount Waverly, Vic, Australia) for 60 min at room temperature (diluted 1:1000 with PBS/BSA). In each case antibodies were used at the concentrations recommended by the provider. Explants were counterstained with propidium iodide (100 ng/ml; Invitrogen) for 2–10 min to visualize cell nuclei. Fluorescent labeling was visualized using a laser-scanning confocal microscope, Axioskop 2 – LSM 5 Pascal (Carl Zeiss, Jena, Germany) and accompanying software.

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