The Imprinted Gene *Peg3* Is Not Essential for Tumor Necrosis Factor α Signaling

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SUMMARY: The imprinted gene *Peg3* encodes a zinc-finger protein which has been proposed to be involved in tumor necrosis factor α (TNF) signaling via an interaction with TNF receptor-associated factor 2 (TRAF2). Primary embryonic fibroblasts derived from mice with a null mutation in *Peg3* showed no abnormalities in TNF-induced nuclear translocation of nuclear factor κB (NF-κB) or phosphorylation of the mitogen-activated protein kinases, extracellular signal-regulated kinases 1 and 2, *c*-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38. In addition, the loss of Peg3 function did not increase the sensitivity of the cells to the cytotoxic action of TNF. These results suggest that Peg3 does not play an essential role in TNF signal transduction. (*Lab Invest 2000, 80:1509–1511*).

*P*eg3 is an imprinted gene which is expressed exclusively from the paternal chromosome (Kuroiwa et al, 1996; Relaix et al, 1996). It encodes a predicted 1572 residue zinc finger protein of unknown function. Peg3 is expressed predominantly in the brain of adult mice with more widespread expression in embryonic tissues (Kuroiwa et al, 1996; Relaix et al, 1996). Relaix et al (1998) recently proposed that Peg3 is intimately involved with the intracellular signal transduction pathways activated by tumor necrosis factor α (TNF). TNF is a pleiotropic cytokine playing a pivotal role in immune and inflammatory regulation, primarily through activation of gene expression via the transcription factors, nuclear factor κB (NF-κB) and activator protein 1 (AP-1). These pathways are critical for the proinflammatory and anti-apoptotic actions of TNF (Baeuerle, 1998; Fiers, 1991; Roulston et al, 1998; van Antwerp et al, 1998). TNF triggers a cascade of intracellular signaling events, after binding to two cell surface receptors (TNF receptors 1 and 2), with subsequent recruitment of cytoplasmic signaling molecules (Ledgerwood et al, 1999). TNF triggers activation of the NF-κB-inducing kinase (NIK) leading to NF-κB activation, phosphorylation of the mitogen-activated protein (MAP) kinases leading to activation of AP-1 (Eder, 1997), and activation of caspases leading to apoptosis (Yuan, 1997). After TNF stimulation, TNF receptor-associated factor 2 (TRAF2) is recruited to the intracellular domains of both TNF receptor 1—via the adaptor molecule TNF receptor-associated death domain protein (TRADD)—and TNF receptor 2. Early reports based on overexpression studies suggested that TRAF2 coupled TNF to NF-κB activation. However, data from TRAF2-null mice has challenged this concept. Thus, cells derived from TRAF2-null mice show only minor abnormalities in TNF-induced NF-κB activation, but complete disruption of TNF-stimulated phosphorylation of *c*-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (Lee et al, 1997; Yeh et al, 1997).

Relaix et al (1998) demonstrated that Peg3 physically interacted with TRAF2, suggesting it could be involved in TNF signaling. In transfection studies, Peg3 activated an NF-κB reporter construct, and a dominant-negative Peg3 construct inhibited TNF-induced activation of NF-κB. Furthermore, Peg3 was reported to protect cells from the cytotoxic effect of TNF.

Recently mice have been generated with a null mutation in *Peg3*. The female mice show aberrant maternal behavior with neglect of pups (Li et al, 1999). To test the hypothesis that Peg3 is involved in TNF signaling, we have used primary embryonic fibroblasts (PEFs) and neuronal cells derived from mice with the *Peg3* null mutation to determine the effect on TNF-induced nuclear translocation of NF-κB, phosphorylation of MAP kinases, and cell death.

Results and Discussion

To assess whether Peg3 is essential for TNF signaling, we used cells derived from mice with the *Peg3* null mutation and compared them with cells derived from wild-type controls. The mutant allele contains the *lacZ* reporter gene, and β-galactosidase staining was used to assess whether particular cell types normally express Peg3. PEFs at passages 2 and 3 (but not later passages) showed clear β-galactosidase staining in the majority of cells, and these cells were used to assess the effect of the null mutation on TNF signaling.
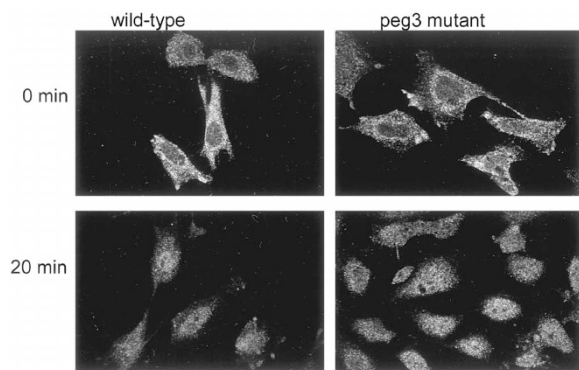
Nuclear Translocation of NF-ϰB in Response to TNF

In unstimulated cells, NF-ϰB is sequestered in the cytoplasm by IϰB. Following activation, IϰB is phosphorylated, polyubiquinated, and degraded, thus unmasking the NF-ϰB nuclear localization signal. Once in the nucleus, NF-ϰB stimulates transcription of a range of TNF-responsive genes. Using immunofluorescence microscopy, we assessed the effect of the loss of Peg3 function on TNF-induced nuclear translocation of NF-ϰB. Wild-type and mutant cells were incubated with TNF for 0 hours, 10 minutes, 20 minutes, 30 minutes, 1 hour, and 2 hours before immunostaining with an anti-NF-ϰB antibody. In both cell types, maximum nuclear translocation occurred at 20 minutes, and at this time point, over 90% of cells showed nuclear localization of NF-ϰB (Fig. 1). There was no apparent difference between the wild-type and mutant cells in the extent of staining at any time point examined. This suggested that Peg3 is not an essential component of the signaling pathway between TNF and the nuclear translocation of NF-ϰB.

The brain is a major site of Peg3 expression in the neonate and adult. Therefore, we also investigated whether there was a neuronal cell-specific loss of TNF-induced NF-ϰB nuclear translocation in primary cells derived from hypothalami of new-born Peg3 mutant mice compared to the wild-type control. Neither wild-type nor mutant neuronal cells, identified by co-staining with an antibody against β-tubulin III, showed nuclear translocation of NF-ϰB in response to TNF. However, nonneuronal cells from both wild-type and mutant animals in the same culture responded identically to TNF (data not shown).

Phosphorylation of MAP Kinases in Response to TNF

The role of TRAF2 in TNF signaling has been reassessed following the generation of TRAF2-null mice (Lee et al, 1997; Yeh et al, 1997). Thus, cells derived from TRAF2-null mice show only minor abnormalities in TNF-induced NF-ϰB activation but complete disruption of TNF-st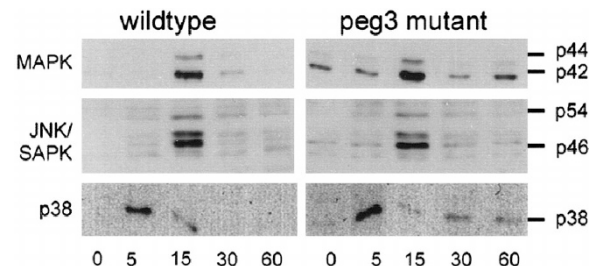imulated phosphorylation of JNK/SAPK. Because Peg3 was reported to interact with TRAF2, we determined whether the loss of Peg3 function in PEFs impaired TNF-induced phosphorylation of the MAP kinases (extracellular signal-regulated kinases 1 and 2), JNK/SAPK, and p38 (Fig. 2). No differences were seen in either the time course or extent of phosphorylation of these protein kinases between the wild-type and mutant cells.

Effect of the Peg3 Null Mutation on the Sensitivity of PEFs to TNF-Induced Cell Death

Although TNF can induce cell death in a variety of cell types, this generally requires the inhibition of ongoing protein synthesis in nontransformed cells (Beyaert and Fiers, 1994). The resistance to TNF-induced killing is thought to be due to activation of anti-apoptotic genes through the transcription factors NF-ϰB and AP-1. If signaling to either of these transcription factors is disrupted, cells may become sensitive to TNF-induced death. Indeed, cells derived from the TRAF2 knockout mice show increased sensitivity to TNF-induced death in the absence of protein synthesis inhibitors (Lee et al, 1997; Yeh et al, 1997).

Wild-type and mutant PEFs were treated with 10 ng/ml TNF, and the extent of cell death was determined by staining with propidium and iodide and fluorescence-activated cell sorting (FACS) analysis. Both cell types were found to be completely resistant to TNF-induced death for up to 48 hours, suggesting that Peg3 is not necessary for protection against the cytotoxic action of TNF.

In summary, in primary embryonic fibroblasts, a cell type which normally expresses Peg3, the absence of Peg3 has no effect on TNF signaling to NF-ϰB and the MAP kinases upstream of AP-1. Furthermore, the *Peg3* null cells are not sensitized to TNF-induced cytotoxicity. These findings do not support the conclusions of Relaix et al (1998) that Peg3 is a regulator of TNF signaling. We cannot rule out the possibility that Peg3 plays a tissue-specific role in TNF responses. Alternatively, the observed interaction between Peg3 and TRAF2, although irrelevant for TNF signaling, may be critical for signaling by other TRAF2-interacting ligand/receptor systems, eg, CD40 (Arch et al, 1998). Our data indicate that Peg3 is not essential for at least three of the key intracellular events mediating the actions of TNF.

Figure 1.
Tumor necrosis factor (TNF)-induced nuclear translocation of NF-ϰB. Wild-type (*left-hand panel*) and mutant (*right-hand panel*) primary embryonic fibroblasts (PEFs) stimulated with 100 ng/ml murine TNF for 0 and 20 minutes and immunostained with rabbit anti-NF-ϰB p65 and a FITC-conjugated secondary antibody.

Figure 2.
TNF-induced phosphorylation of mitogen-activated protein (MAP) kinases. Wild-type (*left-hand panel*) and mutant (*right-hand panel*) PEFs treated with 50 ng/ml mTNF were immunoblotted with anti-ACTIVE MAP kinase, anti-ACTIVE JNK/SAPK, or anti-phospho p38 MAP kinase. Incubation time is in minutes. The sizes of the kinase isoforms in kDa are indicated on the right.

Materials and Methods

Cell Culture

Cells were derived from mice carrying a null mutation in the *Peg3* gene and their wild type controls (Li et al, 1999). Primary embryonic fibroblasts derived from E12.5 embryos were cultured in DMEM/10% fetal bovine serum (FBS) and used at passages 2 and 3. To identify cells containing the *Peg3* null mutation, β-galactosidase staining was performed as described (Li et al, 1999). Cells were isolated from the hypothalami of newborn mice and cultured in DMEM:HamsF12 (1:1)/2% B27/1% FBS on plates coated with poly-L-lysine (0.1 mg/ml). The neuronal cells were identified by immunostaining with anti-mouse-β-tubulin III (Sigma, Pode, Dorset, United Kingdom).

Immunofluorescence Microscopy of NF-$\varkappa$B Nuclear Translocation

Cells were cultured on glass coverslips and stimulated with 100 ng/ml murine TNF (Autogen Bioclear, Calne, Wilts, United Kingdom) in serum-containing medium at 37° C/5% CO_2. After treatment the cells were fixed (2% paraformaldehyde, 4 minutes), permeabilized (100% methanol, 10 minutes, -20° C), and immunostained with rabbit anti-NF-$\varkappa$B p65 (Santa Cruz Biotechnologies, Santa Cruz, California) and an FITC-conjugated secondary antibody (Sigma). Monolayers were viewed using a Nikon Optiphot-II microscope (Nikon, Tokyo, Japan) coupled to a Biorad MRC1000 confocal attachment and COMOS software (BioRad Laboratories, Hemel Hempstead, United Kingdom).

Analysis of MAP Kinase Phosphorylation

Cells were cultured in serum-free medium for 24 hours before treatment with 50 ng/ml mTNF in serum-free medium at 37° C/5% CO_2. After the incubation, the cells were placed on ice and lysed directly into ice-cold sample buffer (62.5 mM Tris.Cl, pH 6.8; 10% glycerol, 2% SDS, 10% 2-mercaptoethanol). Equal amounts were analyzed by immunoblotting with anti-ACTIVE MAP kinase (Promega, Madison, Wisconsin), anti-ACTIVE JNK/SAPK (Promega), or anti-phospho p38 MAP kinase (New England Biolabs, Beverly, Massachusetts). The presence of equal amounts of total kinases in each lane was confirmed by immunoblotting with the appropriate non-phospho-specific antibodies (not shown).

Cell Death Assay

PEFs were treated with 10 ng/ml mTNF in serum-containing medium for up to 48 hours at 37° C/5% CO_2. After the incubation, cells were washed with ice-cold PBS, detached with trypsin/EDTA, and stained with propidium iodide (2 μg/ml in PBS). The proportion of propidium iodide-positive cells was determined by flow cytometry on a FacSort (Becton Dickinson, Lincoln Park, New Jersey).

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