TGF-β modulates programmed cell death in the retina of the developing chick embryo

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SUMMARY

Programmed cell death (PCD) is a key phenomenon in the regulation of cell number in multicellular organisms. We have shown that reduction of endogenous transforming growth factor β (TGF-β) prevents apoptotic PCD of neurons in the developing peripheral and central nervous system, suggesting that TGF-β is an important mediator of ontogenetic neuron death. Previous studies suggested that there are other pro-apoptotic molecules, nerve growth factor (NGF) and brain-derived neurotrophic factor, that induce cell death in the nervous system. In the developing chick retina, NGF induces PCD by activation of the p75 receptor. We have studied the role of TGF-β and its putative interdependence with NGF-mediated PCD in the chick retina. We found that TGF-β is present in the developing chick retina during the period of PCD and is essentially required to regulate PCD of retinal cells. TGF-β2, TGF-β3 and the ligand-binding TGF-β receptor can be detected immunocytochemically in the central retina, a region where apoptosis is most prominent during the early period of PCD. Application of a TGF-β-neutralizing antibody to chick embryos in ovo resulted in a decrease in the number of TUNEL-positive cells and a reduction of free nucleosome levels. In terms of magnitude, reduction of PCD caused by the neutralization of endogenous TGF-β was equivalent to that seen after anti-NGF application. Neutralization of both factors did not result in a further decrease in apoptosis, indicating that NGF and TGF-β may act on the same cell population. Furthermore, neutralization of TGF-β did not affect the expression of NGF or the p75-receptor. Our results suggest that TGF-β and NGF are both required to regulate cell death in the chick retina in vivo.

Key words: TGF-β, NGF, Cell death, Apoptosis, Chick, Retina

INTRODUCTION

Programmed cell death (PCD) occurs as a regulated process during development of multicellular organisms (Jacobson et al., 1997; Metzstein et al., 1998). Within the nervous system, PCD is recognized as a relatively late event in neuron differentiation and maturation that coincides with synaptogenesis of neurons at their target cells (Oppenheim, 1991). According to the neurotrophic factor concept, neuronal survival depends on the capability of their axons to compete for limiting amounts of neurotrophic input from the target area, whereas lack of neurotrophic support results in cell death (Hamburger, 1992). Target-derived neurotrophic factors, such as nerve growth factor (NGF), have been shown to be essential for survival and maintenance of selective developing neurons in vertebrates (Lewin and Barde, 1996; Levi-Montalcini, 1987). The trophic action of NGF is mediated via its ability to activate the receptor tyrosine kinase TrkA (Nebreda et al., 1991; Kaplan et al., 1991). More recently, it has been shown that NGF can also cause cell death during development by solely activating the neurotrophin receptor p75 (p75NTR; Dechant and Barde, 1997; Barker, 1998). Frade and co-workers (Frade et al., 1996) have shown that the induction of cell death by NGF does reflect normal development in vivo, as the application of NGF-neutralizing antibodies reduces PCD in the avian retina.

The developing chick retina undergoes at least two discrete periods of PCD. Particularly in the central retina, the earlier period of PCD has been suggested to serve the purpose of creating space for incoming axons of retinal ganglion cells to form the optic nerve (embryonic day (E) 5-7; Cuadros and Rios, 1988; Martin-Partido et al., 1988), whereas PCD within the later period corresponds to the well-documented process of retinal ganglion cell death after innervation and synapse formation in the optic tectum (E10-E14; Rager, 1980). In the early period, apoptosis is induced by NGF acting via its p75 receptor (Frade et al., 1996; Frade and Barde, 1999). In contrast, brain-derived neurotrophic factor (BDNF) prevents retinal cell death in the early period of PCD (Frade et al., 1997). Both, BDNF and NGF are expressed in the avian retina during development, and their expressions peak around E12-E15 (Hallböök et al., 1996). In the E4 chick retina, blood-borne microglial cells have been identified as the source of death-inducing NGF in the developing eye, indicating an active role for macrophages in neuronal death (Frade and Barde, 1998).
Another growth factor that has been shown to induce PCD in the developing nervous system in vivo is transforming growth factor β (TGF-β; Krieglstein et al., 2000). TGF-β was originally discovered because of its capacity to induce anchorage-independent growth of normal rat kidney cells and fibroblast cells (Moses et al., 1981; Roberts et al., 1981). It soon became apparent that the biological activity of TGF-β was not restricted to this effect on cellular growth. The numerous functions of TGF-β include cell cycle control, regulation of early development, differentiation, extracellular matrix formation, hematopoiesis, angiogenesis, chemotaxis, immune functions and apoptosis (Roberts and Sporn, 1990; Lawrence, 1996; Böttner et al., 2000; Krieglstein et al., 2000; Massague and Chen, 2000). TGF-β is also known as a contextually acting molecule, as its actions often depend on environmental cues, i.e. the cell type, the differentiation state of cells and the presence of other growth factors, best exemplified by its capacity either to stimulate or inhibit proliferation (Nathan and Sporn, 1991; Roberts and Sporn, 1990; Skoff et al., 1998; Ashley et al., 1998).

Three highly homologous, yet distinct isoforms of TGF-β are known from several species including mammals and birds: TGF-β1, TGF-β2 and TGF-β3 (Roberts and Sporn, 1990). TGF-β proteins elicit their cellular responses through formation of heteromeric complexes of specific type I and type II serine/threonine kinase receptors (TβRI, TβRII; for reviews see Itoh et al., 2000; Massague and Chen, 2000). TβRII is a constitutively active kinase, which, upon ligand binding, initiates the signaling cascade that involves phosphorylation of the TβRI, and the propagation of the signal by Smad2 and Smad3. Activated phosphorylation then leads to nuclear translocation of these transcription factors and subsequent transactivation of several target genes (reviewed by Wrana and Attisano, 2000; Itoh et al., 2000).

TGF-βs are expressed during the period of PCD of numerous neuron populations (Krieglstein et al., 1998a; Krieglstein et al., 1998b) including the developing retina. TGF-β is released from cultured retinal pigment epithelial cells (Connor et al., 1988) and is expressed in the ontogenetically related ciliary epithelial cells (Helbig et al., 1991). At later developmental stages, TGF-β2 was found to be the predominant endogenous isoform in the vitreous humor and retinal pigment epithelium (RPE) of monkey eyes (Pfeffer et al., 1994). Immunohistochemical evidence of TGF-β in the retina was first obtained from human eyes, where immunoreactivity was reported to be associated with photoreceptor outer segments (Lutty et al., 1991; Lutty et al., 1993; Pfeffer et al., 1994).

As both NGF and TGF-β have been shown to regulate PCD in vivo, this study addresses the questions of whether TGF-β (like NGF) is required to regulate cell death in the avian retina and, if so, whether NGF and TGF-β are dependent on each other or activate different pathways.

**MATERIALS AND METHODS**

**Embryos**

Fertilized eggs from white Leghorn hens were incubated at 38.5°C in an atmosphere with 70% humidity. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

**Immunoneutralization of TGF-β and NGF in chicken embryos**

Eggs were incubated for 3 days (until stage 19 of development) and were then opened at the blunt pole. The shell membrane was removed and 100 μl phosphate-buffered saline (PBS) solution containing 5 μg anti-NGF (Chemicon), 10 μg neutralizing monoclonal anti-TGF-β 1/2/3 (R&D Systems), or a combination of both, was layered onto the chorioallantoic membrane. In additional experiments, the eggs were treated with 10 μg neutralizing monoclonal anti-TGF-β 1/2/3 (R&D Systems) in combination with 20 μg recombinant NGF (Roche).

The antibodies used are known to block the biological activity of NGF and TGF-β, respectively, as reported elsewhere (Korsching and Thoenen, 1987; Frade and Barde, 1998; Krieglstein et al., 2000). The eggs were sealed and returned to the incubator. This procedure was repeated each 24 hours until the embryos were sacrificed at day 6 (stage 29). Controls were treated with either 100 μl PBS solution or PBS containing 10 μg mouse IgG1 FITC conjugated (DAKO).

Heads of 6-day-old (stage 29) chicken embryos were fixed in Bouin’s fixative (picric acid, formaldehyde and glacial acetic acid) for several hours; the tissue was then dehydrated in a graded series of ethanol and embedded in paraffin wax. In addition, embryos were fixed for 6 hours in PBS/4% paraformaldehyde. After overnight incubation at 4°C in PBS (pH 7.3) containing 30% sucrose, the tissue was embedded in OCT compound (Tissue-Tek) and sectioned at 10 μm using a cryostat.

**Immunohistochemistry**

Paraffin sections (10 μm) were deparaffinized and heated in citrate buffer in a microwave oven to improve antigen retrieval (Jordan et al., 1997). Sections were pre-incubated with 10% normal goat serum (NGS) in PBS containing 0.3% Triton-X 100 for 1 hour. Immunostaining was performed using isoform-specific anti-TGF-β, or anti-TGF-β receptor (TβR) antibodies (TGF-β1, sc-146; TGF-β2, sc-90; TGF-β3, sc-82; TβRI, sc-402; TβRII, sc-400; Santa Cruz) (Flanders et al., 1989) at dilutions of 1:50-1:200. The reaction was visualized (1) with 3,3’-diaminobenzidine (DAB; KEMENTEC), using the rabbit Vectastain Elite ABC avidin-biotin-kit (PK-6101; Vector); or (2) using Cy3- or FITC-conjugated goat anti-rabbit IgG secondary antibodies (ZYMED) at dilution of 1:100-1:200 in 10% NGS/PBS/Triton-X 100.

In addition, we used a polyclonal anti-human p75 antibody (1:100; Promega) to detect the neurotrophin receptor p75 (p75NTR) in the developing chick retina. Reactions were carried out on 10 μm cryosections after preincubation with 10% NGS and visualized using FITC-conjugated or Cy3-conjugated (ALEXA; 1:1000) secondary antibodies.

In all cases, PBS was substituted for the primary antisera as a control, in order to test for nonspecific labeling. No specific cellular staining was observed when the primary antisera was omitted. Additionally, the specificity of the primary antibodies was tested and confirmed by western blotting (data not shown).

Some cross sections of control and anti-TGF-β-treated embryos were counterstained with Hematoxylin and Eosin for morphological studies.

**Acidic phosphatase labeling**

Sections of E4 and E6 embryos were stained with a method previously described (Cuadros et al., 1992). In brief, sections were incubated for 4 hours in the medium described by Namba et al. (Namba et al., 1983) containing 0.01 M sodium tartrate, which reduces or abolishes acidic phosphatase activity in nearly all cells except macrophages (Cuadros et al., 1992). To test the specificity of the histochemical reaction, control sections were incubated in medium without substrate (naphthol AS BI phosphate) or in complete medium containing 0.01 M sodium fluoride, a specific inhibitor of acidic phosphatase activity (data not shown). No enzyme activity was found in these sections.
TGF-β modulates cell death in the chick retina

Quantification of cell death

Cell death was quantified in neural retinae from eye explants applying an enzyme-linked immunosorbent assay (ELISA) (Roche; cat. no. 1544675). This assay allows the quantification of soluble nucleosomes that recognize histones and DNA (Frade et al., 1996; Frade et al., 1997). Retinae of control and treated animals were homogenized in 200 μl PBS and centrifuged at 20,000 g for 10 minutes. A portion of the supernatant was used to quantify proteins by standard methods, and the rest was diluted 1:10 in the buffer provided by the supplier and processed according to the manufacturer’s manual. Absorbence values were normalized with respect to the values obtained with control retinae.

TUNEL staining

As for the immunohistochemistry, heads of 6-day-old chicken embryos were fixed in Bouin’s solution and paraffin-embedded. Sections (10 μm) were deparaffinized and stained with in situ cell death detection kit (Roche) according to the manufacturer’s instructions.

BrdU staining

To test for proliferation, 100 μl BrdU stock solution (10 mM) was administered to control and anti-TGF-β-treated E6 chick embryos. Embryos were sacrificed 4 hours later, the tissue fixed in 4% paraformaldehyde and cryosectioned (see above). Sections were stained with a monoclonal antibody to BrdU, according to the manufacturer’s instructions. (Roche). Cell counts were taken from a defined area (visual field at 20× magnification) in the optic nerve region of control and anti-TGF-β-treated retinae.

RT-PCR

Retinae of three control and three anti-TGF-β treated animals were extracted in 2 ml RLT buffer (Qiagen) and homogenized in a rotor-stator homogenizer. The RNA was extracted according to the manufacturer’s instructions (RNeasy, Qiagen). An additional on-column DNase digestion was performed to avoid cross contamination with genomic DNA. Equal amounts of RNA were reversed transcribed (OmniScript, Qiagen) for 1 hour. 2 μl of the RT-reaction mix were taken for PCR (25 cycles) with NGF- and GAPDH-specific primers (NGF (forward), CGACATCAAAGGCAAAAGG; NGF (reverse), GGCTGCTGTGGGG; GAPDH (reverse), ATCACGCTTCTCCACCTCCC). PCR products were separated in a 2% agarose gel and stained with ethidium bromide.

RESULTS

Localization of TGF-β receptor and TGF-β isoforms in the developing chick retina

We examined the presence of TβRII and TGF-β2 and TGF-β3 in the chick retina during the early period of PCD. At E6, TGF-β2 and TGF-β3 immunoreactivities could be localized mainly in the central retina (Fig. 1C-F). TGF-β2 staining was restricted to the zone of the optic nerve head, a region where apoptosis is most prominent during the early period of PCD (Fig. 1A,C,E). Immunoreactivity for TGF-β3 was likewise associated with the optic nerve. In addition, TGF-β3 was localized in the pigment epithelium, in structures spanning the neuroepithelium, resembling radial Mueller glia cells, and in the presumptive ganglion cell layer (Fig. 1D,F). Labeling for TβRII was distributed evenly over the entire retinal surface, being somewhat more prominent in the optic nerve region (Fig. 1B).

TGF-β regulates cell death in the developing chick retina

To analyse the role of TGF-β during the early period of cell death in the avian retina, neutralizing antibodies, known to neutralize all three chick TGF-β-isofoms (Kriegstein et al., 2000), were applied to the developing chick in ovo during the period E3-E6. We applied the TUNEL assay to detect, quantify and compare apoptotic cell death in control retinae with anti-TGF-β-treated retinae. At E6, TUNEL-positive nuclei were localized across the thickness of the neuroepithelium, with cells being more abundant in the inner half of the retina (Fig. 2A). The distribution of TUNEL-positive nuclei followed a central-to-peripheral gradient, with more labeled nuclei being concentrated at either side of the optic nerve head (Fig. 1A). Retinae from embryos treated with anti-TGF-β-neutralizing antibody showed a remarkable reduction in the number of TUNEL-positive cells when compared with those of control embryos (Fig. 2A,B). The reduction in cell death was quantified by (1) counting TUNEL-positive nuclei on consecutive sections of treated and untreated E6 chick retinae (Fig. 2C); and (2) measuring free nucleosome levels in retinal extracts (by ELISA; Fig. 2D). Both assays revealed that apoptosis is significantly reduced by 40% in retinae obtained from anti-TGF-β-treated embryos when compared with untreated controls (Fig. 2C,D), suggesting that TGF-β is required to induce PCD of retinal neurons during the early period of cell death in vivo.

To compare TGF-β and NGF in terms of their PCD-inducing capacities, chick embryos were also treated with NGF-neutralizing antibodies as described (Frade et al., 1996). As shown in Fig. 2C,D, the reduction in PCD caused by neutralization of NGF was equivalent to that seen after anti-TGF-β-treatment. In order to test whether NGF and TGF-β act on identical, overlapping or distinct populations of cells anti-TGF-β-treatment was combined with anti-NGF-treatment. However, no synergistic effect could be observed in embryos treated with both anti-TGF-β and anti-NGF neutralizing antibodies (Fig. 2C,D), suggesting that NGF and TGF-β are both required to kill retinal cells in vivo.

To investigate whether the application of exogenous NGF blocks the cell death-decreasing effect of the TGF-β-neutralizing antibody, developing chick embryos were simultaneously treated with TGF-β-neutralizing antibody and recombinant NGF. As shown in Fig. 2E the application of NGF did not override the anti-TGF-β effect, resulting in an equivalent decrease in apoptosis.

Increased size of the retina upon anti-TGF-β treatment

Comparison of retinal morphology in HE-stained sections of control and anti-TGF-β-treated chick embryos revealed a substantial change in eye morphology. Retinae of anti-TGF-β treated embryos are (1) much thicker compared with control retinae (Fig. 3B,D), (2) folded and (3) often lose contact to adjacent cell layers (Fig. 3B). Measuring the thickness of the retinae in three random areas of each section (five sections per animal), we found that – even in animals that show less drastic upfolding of the retina – retinae of anti-TGF-β treated animals were about 20 μm thicker than the average retina of control animals (97 μm; P=0.00027).
Increased cell numbers do not result from increased proliferation

We found convincing evidence that cell death was diminished in anti-TGF-β-treated retina, but in view of morphological changes, the question arises of whether this reduction in cell death might be also accompanied by increased cell proliferation.

TGF-β is a well-established inhibitor of proliferation in several systems (Lawrence, 1996; Gold, 1999). To test for alterations in cell proliferation, we applied BrdU to control and anti-TGF-β-treated chick embryos, and studied BrdU incorporation in retinal sections. Fig. 4A-F shows that there was no apparent difference in BrdU incorporation in retinæ of control or anti-TGF-β treated embryos. Quantification of BrdU-positive cells in the central part of the retina (Fig. 4G) confirmed the staining results, suggesting no significant alteration in cell proliferation in response to neutralization of TGF-β. However, cells treated with anti-TGF-β may show some hyperplasia, which could account at least partially for the increased thickness of treated retinæ.

Acidic phosphatase labeling of microglial cells

The effect TGF-β exerts in the developing chick retina during the period of PCD can be explained by (1) a direct action of TGF-β on cells destined to die, or (2) a more indirect mechanism that affects the source of NGF or the expression patterns of NGF or p75 receptor. As TGF-β is known for its chemoattractive actions on macrophages (Krieglstein et al., 1995; Wahl et al., 1987; Pratt and McPherson, 1997), neutralization of TGF-β might prevent the migration of microglial cells into the retina, thereby eliminating the source of NGF, which is necessary for cell death (Frade and Barde, 1998). To test if microglial cells, the established source of NGF, are still present in anti-TGF-β-treated animals, we stained microglial cells with a previously described histochemical method that detects acidic phosphatase activity (Cuadros et al., 1992). The staining technique includes the use of an inhibitor (sodium tartrate) that abolishes the enzymatic activity in most cells, except macrophages. The product of this enzymatic activity yields a red product (Fig. 5). In sections incubated in medium without substrate or in medium with 0.01 M sodium fluoride, none of the cells was positively stained (Fig. 5A,C). At all stages analyzed (E4, E5 (data not shown) and E6 (see Fig. 5)), intensely stained acidic phosphatase-positive cells were seen on the vitreal surface, in both untreated (Fig. 5C,E) and anti-TGF-β-treated retinae (Fig. 5B,D). Shape and location of these cells resemble those shown by Cuadros et al. (Cuadros et al., 1992) and Frade and Barde (Frade and Barde, 1998), indicating that these cells are microglial cells. These data indicate that microglial cells, the cellular source of NGF in the developing retina, are still present in anti-TGF-β-treated embryos.

Neutralization of TGF-β does not affect NGF and p75 expression

In a previous report (Frade and Barde, 1998) it has been shown that all cells positive for acidic phosphatase activity are also positive for NGF immunoreactivity in the chick retina. Although anti-TGF-β treatment did not affect the appearance and migration of microglial cells, it may still influence the expression of NGF and/or its receptor p75, thereby abolishing cell death mediated by this pathway. As no anti-NGF antibody suitable for immunostaining in chick tissue was available to us, we analysed the presence of NGF using RT-PCR. Results shown in Fig. 6 indicate that equal levels of NGF expression are detectable in retinae of both control and anti-TGF-β-treated animals (lanes 4 and 5). A parallel test for GAPDH confirmed that equal amounts of RNA were used in the RT reaction (lanes 9 and 10). PCR reaction with crude RNA preparation revealed no amplification product (lane 2 and 3, 7 and 8), which excluded cross contamination with genomic DNA.
Staining for p75NTR with a p75 receptor-specific antibody revealed no changes in the expression pattern of the receptor in anti-TGF-β-treated retinae compared with control retinae (Fig. 7A-C). To reveal a possible colocalization of both receptors, staining of consecutive cryosections with p75NTR receptor- and TβRII-specific antibodies were performed. As shown in Fig. 8A, virtually all cells are TβRII-positive. The staining pattern for p75NTR receptor (Fig. 8B) mainly overlaps with TβRII expression in the prospective ganglion cell layer and the optic nerve region, the main area of programmed cell death. Together, these data suggest that neutralization of TGF-β neither abolishes nor downregulates the NGF-dependent extracellular apoptotic pathway.

**Neutralization of NGF does not affect TGF-β and TβRII expression**

If TGF-β does not act upstream of NGF (i.e. it does not change NGF-related parameters), NGF may be upstream of TGF-β. We therefore tested for the presence of TGF-β isoforms and TGF-β receptor in anti-NGF-treated retinae of E6 chicken. However, immunoreactivity for TGF-β2, TGF-β3 and TβRII was found in identical distributions in anti-NGF-treated chick embryos (Fig. 9) and control animals (see Fig. 1).

**Conclusion**

Neutralization of TGF-β reduces cell death in the chick retina in vivo, as does neutralization of NGF. Apparently, neutralization of TGF-β does not affect NGF or p75, its cell death receptor. The cell death-inducing capacity of NGF may depend on the presence and function of TGF-β and vice versa, possibly at least the level of signal transduction. The execution of PCD in retinal cells in vivo may therefore be regulated by both factors: NGF and TGF-β.

**DISCUSSION**

The developing chick retina is an easily accessible system to investigate the role of neurotrophic factors thought to be involved in the regulation of cell survival and death (Cuadros and Rios, 1988; Frade et al., 1996). We analyzed the early period of developmental cell death in the chick retina as this period has been used to demonstrate the role of NGF to induce cell death in the developing retina in vivo (Frade et al., 1996). Our results indicate that endogenous TGF-β is required to kill retinal cells in vivo. Neutralizing antibodies to TGF-β, NGF, or both, reduce cell death to a similar extent, suggesting that TGF-β and NGF are both essential to induce cell death, and that both factors act either consecutively or signal into the same executing system.

**Cell death in the chick retina and localization of endogenous TGF-β**

TGF-β receptor (TβRII), TGF-β2 and TGF-β3 were detected immunocytochemically in the optic nerve region of the developing central chick retina at E6. This staining pattern overlaps with the region where cell death is most prominent in the early phase of PCD (Frade et al., 1997; Cuadros and Rios, 1988). In addition, TGF-β3 staining was localized in retinal pigment epithelium (RPE) cells, in structures spanning the neuroepithelium, resembling Müller glial cells and fibers, and in the presumptive retinal ganglion cell (RGC) layer. TβRII is distributed evenly over the retinal surface and essentially expressed in all cell types. Its distribution overlaps with p75 expression in the optic nerve region and the presumptive ganglion cell layer (compare Fig. 1B with Fig. 7). Our results resemble the findings of Frade...
and Barde, who showed p75 expression in the RGC layer and optic nerve of E15.5 mice (Frade and Barde, 1999).

Localization of TGF-β receptor and TGF-β isoforms in the optic nerve region, an area where apoptosis is most prominent during the early period of PCD strongly suggests that TGF-β plays a role in the regulation of ontogenetic cell death.

Reduction of endogenous TGF-β prevents PCD in the developing chick retina

In the present study, neutralizing endogenous TGF-β by application of an anti-TGF-β antibody to developing chick embryos in ovo (E3 to E6) resulted in a substantial reduction of cell death revealed (1) by counting TUNEL-positive cells in the central retina and (2) comparing the amount of free nucleosome levels in retinal extracts (ELISA). These data are in accordance with the pro-apoptotic role of endogenous TGF-β during ontogenetic neuron death in vivo, most recently demonstrated by Kriegstein et al. (Kriegstein et al., 2000). In this study, PCD of chick ciliary, dorsal root and spinal motoneurons was largely prevented after application of a neutralizing antibody that recognizes all three TGF-β isoforms. Likewise, preventing TGF-β signaling by blocking the TGF-β receptor II during the period of PCD in the ciliary ganglion rescued all neurons that normally die. TUNEL staining revealed decreased numbers of apoptotic cells after TGF-β antibody treatment, whereas application of exogenous TGF-β was able to rescue the TGF-β-deprived phenotype (Kriegstein et al., 2000).

The substantial changes in eye morphology seen in anti-

TGF-β-treated chick embryos strongly resembles retinal phenotypes of Apaf1 and caspase 3 mouse mutants (Pettmann and Henderson, 1998; Cecconi, 1999; Nicholson and Thornberry, 1997). Both molecules are acknowledged executioners of the apoptotic cascade and, similar to our observations, retinae of homozygous E12.5 Apaf1 mutants are noticeably thicker. At E14.5, the hyperplasic retina occupies most of the eye cup and is folded (Cecconi et al., 1998). Moreover, caspase 3-deficient mice that die perinatally with massive cell overgrowth in the CNS (as a result of apoptosis deficiency), likewise exhibit retinal hyperplasia. There are protrusions and indentations of the retinal neuroepithelium, causing compression on the lens (Kuida et al., 1996). Cross sections of retinae from caspase 3 mutants closely resemble the phenotype of anti-TGF-β-treated chick embryos (Fig. 3B). Together, these data further add to the notion that the phenotype detected in the developing chick retina after
TGF-β modulates cell death in the chick retina

However, as TGF-β may also effect extracellular matrix assembly (Roberts et al., 1992), folding and detachment of the retina in anti-TGF-β-treated chick embryos may result from an independent effect.

Comparison with NGF-induced cell death

As the anti-TGF-β-dependent reduction of apoptotic cell death in the chick retina was comparable with that induced by application of NGF neutralizing antibodies (Frade et al., 1996; Fig. 2), it was important to elucidate the selective significance of endogenous TGF-β is due to decreased PCD. However, as TGF-β may also effect extracellular matrix assembly (Roberts et al., 1992), folding and detachment of the retina in anti-TGF-β-treated chick embryos may result from an independent effect.

Fig. 5. Acidic phosphatase labeling of microglial cells in the developing E4 (B,C) and E6 (D,E) chick retina. The product of this enzymatic activity can be detected by a histochemical method that yields a red product. Intensely stained acidic phosphatase-positive cells are seen on the vitreal surface of anti-TGF-β-treated (arrowheads in B,D) and untreated retinae (arrowheads in C,E). No enzyme activity was found in control sections incubated in medium without substrate (naphtol AS BI phosphate; A,C). Scale bar: 20 μm.

Fig. 6. Expression of NGF in the E6 chick retina. RT-PCR with NGF- and GAPDH-specific primers was performed as described (see Materials and Methods). The PCR products were separated on a 2% agarose gel and stained with ethidium bromide. NGF is detectable in both control and anti-TGF-β-treated retinae. NGF, lane 4 (control) and lane 5 (anti-TGF-β); GAPDH, lane 9 (control) and lane 10 (anti-TGF-β). PCR with crude RNA preparations revealed no amplification product (lanes 2,3,7,8), ruling out cross contamination with genomic DNA. M, marker lane.

Fig. 7. Expression of p75NTR in the E6 chick retina. p75NTR immunoreactivity was observed in the prospective ganglion cell layer and optic fiber layer (arrowheads in A-C) of control (A) and anti-TGF-β-treated (B,C) retinae. Staining was also detectable in the optic nerve (arrows in C). nr, neural retina; on, optic nerve; pe, pigment epithelium. Scale bar: 20 μm.
of each of the two growth factors. As co-application of NGF- and TGF-β-neutralizing antibodies did not result in any further reduction of cell death, we conclude that both molecules, TGF-β and NGF, act on the same populations of cells. If the pathways were clearly different, NGF would block the anti-TGF-β effect. However, simultaneous application of both, TGF-β neutralizing antibody and recombinant NGF did not affect the reduction in apoptosis evoked by TGF-β neutralization. One might interpret these data as a hint that the biological response to NGF depends into a common downstream pathway. NGF may act consecutively, or may signal by feeding by microglial cells (Frade and Barde, 1998). Mechanistically, it is conceivable that TGF-β and NGF may act consecutively, or may signal by feeding into a common downstream pathway.

One possibility concerning TGF-β acting upstream of NGF may be a chemoattractant role of TGF-β (Wahl et al., 1987; Yao et al., 1990; Pratt and McPherson, 1997) for microglial cells. NGF-presenting blood-borne microglial cells have been shown to be the cellular source of NGF and to mediate the NGF-dependent cell death (Frade and Barde, 1998). However, the failure to detect any apparent differences in distribution of microglial cells, retinal localization of NGF or p75NTR expression in anti-TGF-β-treated retinas compared with untreated retinas suggests that endogenous NGF is not sufficient to induce retinal cell death on neutralization of TGF-β. Alternatively, if TGF-β is not upstream of NGF (i.e. it does not change NGF-related parameters), NGF may act upstream of TGF-β. We therefore tested for changes in the expression pattern of TGF-β and receptor in anti-NGF treated E6 chick retinae and found an identical localization of TGF-β2 and TGF-β3 as well as TβRII, suggesting that NGF and TGF-β do not act consecutively, but are both required to induce cell death.

TGF-β is well known as a contextual acting molecule (Unsicker and Krieglstein, 2000; Nathan and Sporn, 1991), which means that many functions of TGF-β are dependent on the presence of other growth factors. For example, TGF-β acts synergistically with established neurotrophic factors to promote survival of many neuron populations (Krieglstein et al., 1995; Krieglstein et al., 1998a; Krieglstein et al., 1998b). TGF-β synergizes with glial-derived neurotrophic factor to promote survival of peripheral and CNS dopaminergic neurons in vitro and in vivo (Krieglstein et al., 1998b; Schober et al., 1999). With regard to the regulation of apoptosis, TGF-β has been shown to interact cooperatively with tumor necrosis factor α (TNFα) to induce cell death in Schwann cells in vitro (Skoff et al., 1998). Neither TNFα nor TGF-β alone is capable of inducing cell death in these cells alone, despite the fact that both growth factors induce cell death in a variety of different cell types (Smyth and Johnstone, 2000; Wahl et al., 2000; Gold, 1999). Taken together, the present study provides the first evidence to suggest that TGF-β and NGF cooperate in inducing developmental cell death in the central retina in vivo. Notably, the TNFα receptor belongs to the same family of death receptors as p75NTR (Casaccia-Bonnefil et al., 1999). One might therefore speculate that under certain circumstances – during embryonic development – signaling through this receptor family may require TGF-β to induce cell death.

**Fig. 8.** p75NTR (A) and TβRII (B) immunoreactivity in consecutive cryosections of an E5 chick retina, as revealed by indirect fluorescent stains (p75NTR, FITC/green; TβRII, CY3/red). TβRII immunoreactivity is distributed evenly, whereas p75NTR staining is mainly observed in the prospective ganglion cell layer and optic fiber layer, as well as in the optic nerve. The main overlap of both receptors can be found in the optic nerve region, where most cells stain positively for both, p75NTR and TβRII. on, optic nerve; ov, optic ventricle. Scale bar: 50 μm.

**Fig. 9.** Expression of TGF-β isoforms (B,C) and receptor (TβRII; A) in anti-NGF-treated E6 chick retinas, revealed by indirect immunofluorescence stains. As in control retinae, TβRII immunoreactivity is distributed evenly over the entire retinal surface (A), whereas TGF-β2 (B) and TGF-β3 (C) immunoreactivity was observed mainly in the central retina in the region around the optic nerve head. In addition, TGF-β3 is localized in structures that span the neuroepithelium, resembling Müller glia cells or radial Müller fibers (arrowheads in C), and in the presumptive ganglion cell layer (arrow in C). nr, neural retina; on, optic nerve; ov, optic ventricle; pe, pigment epithelium. Scale bar: 50 μm.
We have shown for the first time that endogenous TGF-β is required for cell death occurring in the developing chick retina in vivo, which was previously attributed to NGF. Our results strongly suggest that both NGF and TGF-β cooperate in the regulation of PCD.

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