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Embryonic Mouse Submandibular Salivary Gland Morphogenesis and the TNF/TNF-R1 Signal Transduction Pathway

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ABSTRACT

TNF is a pleiotropic cytokine that modulates cell proliferation and apoptosis. The objective of the present study was to investigate the possible function(s) of the TNF/TNF-R1 signaling pathway in embryonic mouse submandibular salivary gland (SMG) morphogenesis. After characterizing in vivo mRNA and protein expression of various constituents of this pathway, we utilized in vitro experiments to investigate the phenotypic outcomes of enhanced and deficient ligand. The results of these experiments indicate that the TNF/TNF-R1 signal transduction pathway plays an important role in balancing cell proliferation and apoptosis during SMG duct and presumptive acini formation. Anat Rec 262:318–330, 2001.

Key words: embryonic; submandibular salivary gland; mouse; TNF; TNF-R1; NFκB; cell proliferation; apoptosis

Embryonic mouse submandibular salivary gland (SMG) branching morphogenesis is best conceptualized in stages (Jacksoll and Melnick, 1999): 1) *Initial Bud Stage*: primitive oral cavity epithelium adjacent to the developing tongue grows down into compact mesenchyme to form a solid, elongated epithelial stalk terminating in a bulb; 2) *Pseudoglandular Stage*: the solid cord of epithelia elongates and grows by repeated end-bud branching into the surrounding mesenchyme; 3) *Canalicular Stage*: the number of lobes is increased, the presumptive ducts begin to exhibit distinct lumina lined by cuboidal epithelial cells, and the mesenchyme is more loosely packed ; 4) *Terminal Bud Stage*: distinct, well-developed lumina are seen in presumptive ducts and terminal end buds (presumptive acini). Epithelial cell proliferation is found in all stages, even after well-defined lumen formation in the *Terminal Bud Stage*(Jaskoll and Melnick, 1999). Epithelial cell apoptosis begins with the onset of lumen formation in the *Canalicular Stage*.

Progressive SMG branching morphogenesis is mediated by hormones, growth factors, cytokines, and the like in such a way as to translate endocrine, autocrine, and paracrine signals into specific gene responses regulating cell division, apoptosis, and histodifferentiation (Melnick and Jaskoll, 2000). Recently we reported the unique spatiotemporal protein expression in embryonic SMGs of four signal transduction pathways: TGF-α/EGF, IGF, TGF-β, and TNF (tumor necrosis factor) (Jaskoll and Melnick, 1999). TNF/TNF-R1 (TNF receptor 1) expression was associated with sites of ductal and presumptive acini lumina formation, sites that exhibit both epithelial cell proliferation and apoptosis.

TNF is a multifunctional cytokine that transduces its signal through two distinct TNF receptors, TNF-R1 and TNF-R2. Although most tissues, including SMG epithelium, coexpress both receptors, cellular responses to soluble TNF seem to be primarily effected through TNF-R1 because of the marked stability of TNF/TNF-R1 complexes in contrast to the transience of TNF/TNF-R2 complexes

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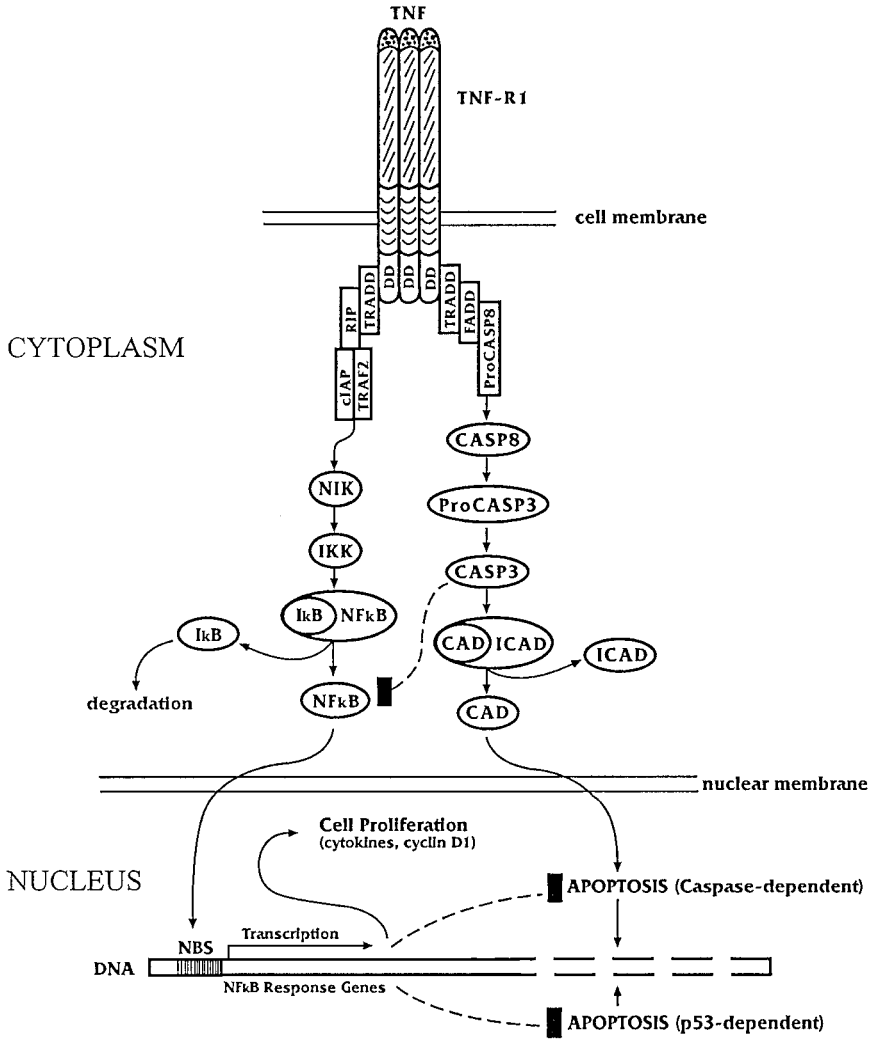


Fig. 1. Summary of the TNF/TNF-R1 signal transduction pathway. Abbreviations: CAD, caspase-activated DNase; CASP, caspase; cIAP, cellular inhibitor of apoptosis protein; DD, death domain; FADD, Fas associated death domain protein; ICAD, inhibitor of CAD; IκB, inhibitory κB; IKK, IκB kinase; NBS, NFκB binding site; NFκB, nuclear factor-κB; NIK, NFκB-inducing kinase; ProCASP, precursor caspase; RIP, receptor interacting protein; TNF, tumor necrosis factor; TNF-R1, tumor necrosis factor receptor 1; TRADD, TNF-R1 associated death domain protein; TRAF2, TNF-R associated factor (Darnay and Aggarwal, 1997; Natoli et al., 1997; Yuan et al., 1997; Ashkenazi and Dixit, 1998; Liu et al., 1998; Van Antwerp et al., 1998; Wang et al., 1998; Zörnig et al., 1998; Guttridge et al., 1999; Levkau et al., 1999; Wallach et al., 1999; Webster and Perkins, 1999). Solid arrows represent promotive pathways; dashed lines terminating at a closed box represent inhibitive pathways.

(Grell et al., 1998). TNF-R1 lacks intrinsic signaling capacity and transduces signals by recruiting associating molecules specific to the downstream functions of modulating cell proliferation and apoptosis (Fig. 1) (Darnay and Aggarwal, 1997; Natoli et al., 1997; Yuan, 1997; Ashkenazi and Dixit, 1998; Wallach et al., 1999). TNF binds to the extracellular domain of the TNF-R1 and induces receptor trimerization and aggregation of the cytoplasmic "death domain" (DD). DD recruits the adapter protein TRADD that in turn recruits FADD and pro-Caspase 8 to initiate the apoptosis pathway and RIP, cIAP, and TRAF2 to initiate the NFκB pathway. In most circumstances, the NFκB pathway is dominant (Ashkenazi and Dixit, 1998).

TNF has been shown to have a marked effect on the growth and differentiation of other branching organs. TNF supplementation of developing rat mammary glands in vitro significantly enhances lobular and ductal morphogenesis and casein production (Ip et al., 1992). Similarly, TNF in vitro supplementation of embryonic lung primordia has a dose-dependent, stimulating effect on epithelial branching and surfactant-associated protein expression (Jaskoll et al., 1994a). These studies complement the accumulating evidence that TNF is commonly present during normal embryogenesis (Ohsawa and Natori, 1989; Gendron et al., 1991; Deman et al., 1992; Wride and Sanders, 1993).

The objective of the present study was to investigate the possible function(s) of the TNF/TNF-R1 pathway in embryonic mouse SMG morphogenesis. After characterizing in vivo mRNA and protein expression of various constituents of this pathway, we utilized in vitro experiments to investigate the effects of enhanced and deficient ligand.

Results of these experiments indicate that the TNF/TNF-R1 signal transduction pathway is important to regulation of the delicate balance between cell proliferation and apoptosis in SMG duct and presumptive acini formation.

MATERIALS AND METHODS

Tissue Collection

Female B10A/SnSg mice, obtained from Jackson Laboratories (Bar Harbor, ME), were maintained and mated as previously described (Jaskoll et al., 1994b); plug day = Day 0 of gestation. Pregnant females were anesthetized on Days 13–18 of gestation (E13–18) with methoxyflurane (metafane) and euthanized by cervical dislocation. Embryos were dissected in cold phosphate buffered saline (PBS) and staged according to Theiler (Theiler, 1989). SMGs were dissected and cultured, processed for histology, or stored at −70°C. For RNAse protection studies, maternal liver and spleen were also collected, cut into 2 mm pieces, and stored at −70°C.

Culture System

E13 or E15 SMG primordia were cultured using a modified Trowell method as previously described (Jaskoll et al., 1994b). The medium consisted of BGJb (Life Technologies, Rockville, MD) supplemented with 0.5 mg ascorbic acid/ml and 50 U penicillin/streptomycin (Life Technologies), pH 7.2, and replicate cultures were changed every other day. Cultures were supplemented on Day 0 with 10 U/ml recombinant mouse TNF (rTNF, R & D, Minneapolis, MN) and maintained for the duration of the experiments; this dose had previously been shown in our laboratory to induce a significant increase in mean branch number. To evaluate the affect of TNF on early epithelial branching, E13 primordia were cultured in control or TNF-supplemented media for 3 days (E13 + 3) and assayed for branching morphogenesis according to method of Spooner as previously described (Jaskoll et al., 1994b). Briefly, the number of lobes per explant was counted on Days 1 and 3 of culture and changes in the complexity of branching morphogenesis were expressed as ratios of branching (t_{72}/t_{24}). The experimental group consisted of 6 control and 7 TNF-treated explants. Mean Spooner ratios for TNF-treated explants were compared with controls as described below. To evaluate the affect of TNF on *Canalicular Stage* SMGs, E15 primordia were cultured for 4 days (E15 + 4) with or without TNF supplementation, examined with a dissecting microscope at time of collection, and processed for epithelial morphogenetic or cell proliferation analyses as described below. Four independent experiments were conducted, consisting of a minimum of 10 TNF-treated and 10 control explants per experiment. To determine if TNF supplementation induces SMG acinar-specific mucin protein expression, E15 primordia were cultured for 4 days (E15 + 4) or 7 days (E15 + 7) with or without TNF supplementation and evaluated for mucin protein expression by immunohistochemistry; 3 independent experiments were conducted, each consisting of 3 TNF-treated and 3 control explants per experiment.

Inhibition Studies

To determine the role of endogenous TNF during embryonic SMG development, we conducted thalidomide (TH)-induced, TNF inhibition experiments. We initially cultured E15 primordia in the presence of 10 or 20 μg/ml thalidomide (Celgene Corporation, Warren, NJ) dissolved in BGJb medium containing 0.05% DMSO, control BGJb medium containing 0.05% DMSO (CONT-DMSO), or control BGJb (CONT). The concentrations used were two to four times the concentration previously shown to suppress TNF expression in cell lines (Shannon et al., 1997). Three independent experiments were conducted, each consisting of a minimum of eight TH-treated explants per concentration, eight CONT-DMSO, and eight CONT explants. Explants were evaluated with a dissecting microscope at time of collection and processed for histological examination; SMG morphogenesis was evaluated in a minimum of six explants per group. After determining that 20 μg/ml effected a response in embryonic SMGs, 20 μg/ml TH was then used in all subsequent studies. We then conducted four independent experiments, with a minimum of eight CONT, eight CONT-DMSO or eight TH-treated explants per experiment. To determine if TNF supplementation could induce SMG recovery from the TH effect, E15 primordia were cultured for 4 days in the presence of 10 U/ml rTNF + 20 μg/ml TH and compared with CONT, CONT-DMSO, rTNF-treated, and TH-treated explants. Three *rescue* experiments were conducted, each consisting of a minimum of five explants per group. SMG morphogenesis was evaluated in a minimum of five explants per experimental group by routine light microscopy.

Histology and Immunochemistry

SMGs were fixed in Carnoy's fixative, processed, embedded in low-melting point paraplast, and stored for brief periods at 4°C. For immunochemistry, the tissues were sectioned at 7 μm, placed on cleaned, gelatin-coated slides at 37°C for 3 hr, and immediately immunostained as previously described (Jaskoll et al., 1994b). For rabbit polyclonal antibodies (anti-NFκB p65, IκB-α, mucin), the sections were incubated overnight with the primary antibody and then sequentially incubated in biotin-labeled goat anti-rabbit IgG (Organon Teknika, Durham, NC) and FITC-labeled streptavidin (Zymed, South San Francisco, CA). For goat polyclonal antibodies (anti-TNF-α, TNF-R1, TNF-R2, p53; NFκB p50), the sections were incubated overnight with the primary antibody and then incubated with FITC-labeled anti-goat IgG (Sigma, St. Louis, MO). Because the transcription factor NFκB consists of two subunits, p50 and p65, we used both a goat polyclonal antibody to NFκB p50 and a rabbit polyclonal antibody to NFκB p65 to verify NFκB's distribution; the pattern of immunolocalization was identical for both antibodies (data not shown). Therefore, we only present the spatial distribution of anti-NFκB p65 antibodies in the Results section. To evaluate caspase 8 and activated caspase 3 spatiotemporal distribution, sections were preincubated in 3% hydrogen peroxide before overnight incubation with the primary antibody. The next morning, the sections were sequentially incubated in biotin-labeled goat anti-rabbit IgG (Organon Teknika, Durham, NC), streptavidin-peroxidase (Zymed), DAB substrate (Zymed) and counterstained with hematoxylin. In all experiments, negative controls were incubated in the absence of primary antibody or with preimmune serum; controls were routinely negative. A minimum of four SMGs were evaluated for each stage of development per experimental group.

Antibodies

Polyclonal antibodies to TNFα, TNF-R1, TNF-R2, p53, caspase 8, IκBα, NFκB p50, NFκB p65 were purchased from Santa Cruz Biotech, Inc. (Santa Cruz, CA). Polyclonal rabbit anti-active caspase 3 antibody was purchased from Pharmingen (San Diego, CA). Polyclonal rabbit anti-mouse SMG mucin protein was generated and characterized by Zymed.

Cultured explant morphogenesis was analyzed by light microscopy of serial sections stained with hematoxylin and eosin. For all experimental groups, a minimum of five explants per group was evaluated.

Proliferation Assay

Sections were incubated with anti-PCNA using the Zymed mouse PCNA kit and then counterstained with hematoxylin. For cell proliferation quantitation, three sections per group were selected and three areas per section was photographed at 200×. PCNA-positive epithelial cells/total epithelial cells were determined per area and the mean ratio PCNA-positive epithelial cells per section determined. Cell proliferation of 15 + 4 explants were conducted for CONT, TNF-treated, CONT-DMSO, and TH-treated explants. Because no significant difference between CONT and CONT-DMSO explants was determined, however, comparisons were made between CONT and TNF-treated E15 + 4 explants or CONT and TH-treated E15 + 4 explants were made.

Apoptosis Assay

Apoptotic cells were detected using a monoclonal antibody to single-stranded DNA (ssDNA) (Mab F7-26) according to the method of Apostain, Inc (Miami, FL). Selective binding to anti-ssDNA monoclonal antibody F7-26 to apoptotic nuclei reflects decreased stability of DNA to thermal denaturation. Four positive and negative controls were conducted. *Negative controls*: (1) tissue sections were heated and treated with S1 nuclease (Sigma); S1 nuclease eliminates staining of apoptotic cells, thus demonstrating that Mab F7-26 binds specifically to ssDNA. (2) Sections were pretreated in PBS containing lysine-rich histone (Sigma) before heating and immunostaining; reconstitution with histone restores DNA stability in apoptotic nuclei, thus preventing DNA denaturation and eliminating Mab staining of apoptotic cells. *Positive controls*: (1) sections were heated in water and treated with Mab; bright staining of all non-apoptotic nuclei with low apoptotic indexes demonstrates that the procedure is adequate to detect ssDNA. (2) Sections were pretreated with proteinase K before heating; intensive staining of non-apoptotic cells demonstrates the procedure detects decreased DNA stability induced by the digestion of nuclear proteins. Mab F7-26 was purchased from Apostain, Inc. Apoptosis was evaluated in a minimum of four explants per experimental group. Quantitation of apoptosis was conducted as described above for cell proliferation. Apoptosis is presented as the ratio of apoptotic-positive epithelial cells/total epithelial cells. Mean ratios per section and mean ratios per group were determined. Comparisons between CONT-DMSO and TH-treated E15 + 4 explants were conducted.

mRNA Quantitation

Total RNA was isolated from a minimum of three litters of E14, E15, E17, and E18 SMGs (Jaskoll et al., 1998). RNase protection assay was conducted using a custom Riboquant Multiprobe Apoptosis kit (Pharmingen, San Diego, CA) to evaluate the steady state levels of Caspase-8, FasL, Fas, TNF, IL-6, FAF, TRAIL, TNF-R1, TRADD, RIP transcripts; L32 and GAPDH housekeeping mRNA levels were also determined in each sample to normalize the results. Riboprobes were generated according to the Riboquant protocol and ^{32}P-riboprobes (3×10^6 counts/μl, final concentration 4×10^5) were hybridized with 10 μg total cellular RNA according to the Riboquant protocol. Transcript-protected ^{32}P-labelled RNAs were isolated by phenol-chloroform extraction, recovered by ethanol precipitation, resolved by gel electrophoresis (5% polyacrylamide, 8 M urea), displayed on film by screen-aided autoradiography, and quantified by phosphor imaging as previously described (Jaskoll et al., 1998). All results were normalized to the amount of L32 mRNA in each sample. Three independent experiments were conducted per experimental group.

TNF Protein Determination

Three independent culture experiments were conducted to determine if TH treatment downregulates TNF protein expression, each group consisting of six explants or E15 starting primordia. Eighteen E15 SMG primordia, CONT/DMSO and TH-treated E15 + 4 explants were homogenized with tissue lysate buffer as previously described (Jaskoll et al., 1998) and 2 μl of different concentrations of SMG protein (1 mg/ml, 2 mg/ml, 4 mg/ml and 8 mg/ml) was added to each well onto a nitrocellulose membrane using a dot-blot apparatus (Schleichner Schnell, Keene, NH); this method ensures consistent protein bonding to the membrane by allowing the protein to bind for 1 hr at room temperature. The membrane was initially incubated in Blotto Blocking Solution (Piece, Rockford, IL) overnight at 4°C and then incubated with goat-anti mouse TNF (Santa Cruz) or control goat IgG (Sigma) overnight at 4°C. The next morning, the membrane was sequentially incubated in rabbit anti-goat IgG (Sigma), horseradish peroxidase (HRP)-conjugate mouse anti-rabbit IgG, and enhanced chemiluminescence detection solution (Amersham, Arlington Heights, IL). The membranes were then placed on Hyperfilm ECL (Amersham) and immediately developed. The level of TNF protein was determined by densitometry and expressed as arbitrary densitometry units. The data was determined to be linear for each experimental group.

Statistical Analysis

Means and variances were analyzed utilizing *t*-test, analysis of variance, analysis of covariance, and multiple regression, as appropriate and in the usual manner (Sokal and Rohlf, 1981). To meet the assumptions of these analyses, namely normality and homoscedasticity, counts, ratios, and percentages were log or arcsin transformed (Sokal and Rohlf, 1981).

RESULTS

Developmental Expression of the TNF Signal Transduction Pathway

*m*RNA quantitative analysis.

To investigate the expression in developing SMGs of genes relevant to TNF/TNF-R1 signal transduction, we used a method that permitted us to measure mRNA levels

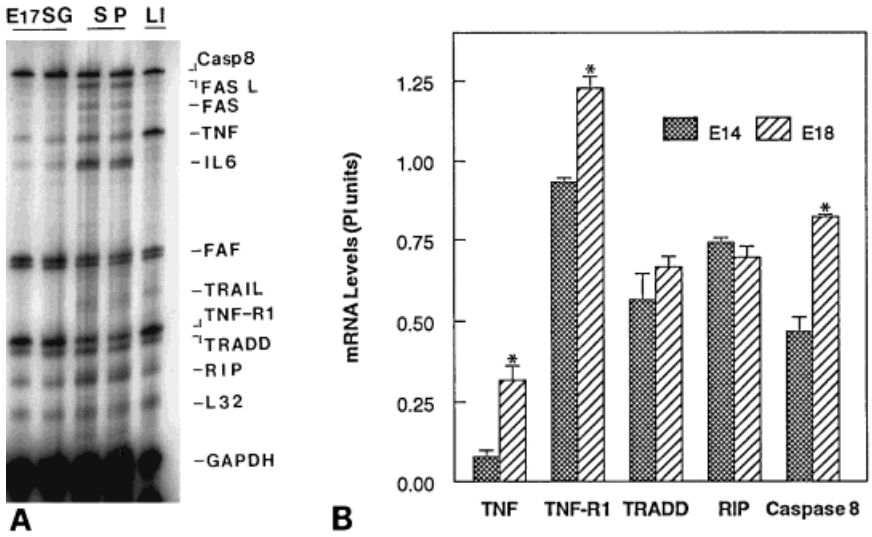


Fig. 2. mRNA quantitative analysis. **A:** Representative multi-probe ribonuclease protection assay, including E17 SMG, adult spleen (SP), and adult liver (LI). **B:** Bar graph of means ± SEM for five relevant transcription products in E14 and E18 SMGs; n = 3 for each bar. Highly significant differences between gestational ages were found for TNF, TNF-R1, and Caspase 8; *$P < 0.01$.

TABLE 1. mRNA quantitation: correlation matrix

	TNF	TNF-R1	TRADD	RIP	Caspase 8	GA[b]
TNF	—					
TNF-R1	0.75[a]	—				
TRADD	0.50	0.66	—			
RIP	0.23	0.52	0.54	—		
Caspase 8	0.88[a]	0.89[a]	0.69	0.26	—	
GA	0.86[a]	0.94[a]	0.55	0.25	0.94[a]	—

[a]Correlations which are highly significant ($P < 0.01$) and have coefficients of determination greater than 50%.
[b]GA, gestational age (E14 → E18).

for multiple genes simultaneously in each independent sample (see Materials and Methods). These included TNF, TNF-R1, TRADD, RIP, Caspase 8, Fas, FasL, FAF, and TRAIL. The choice of TNF, TNF-R1, and TRADD are obvious because they are common to both the NFκB and apoptosis cascades (Fig. 1). RIP and Caspase 8 mRNA were measured because they are the sine qua non of their respective cascades (Fig. 1) (Ashkenazi and Dixit, 1998). We chose Fas, FasL, FAF, and TRAIL because as members of the TNF/TNF-R1 superfamily they utilize a similar, if not identical, repertoire to effect apoptosis (Ashkenazi and Dixit, 1998). Quantitation of mRNA was made on three independent samples for each of 4 gestational days (E), 14, 15, 17, 18. Measurable mRNA was found for TNF, TNF-R1, TRADD, RIP, Caspase 8, and FAF; none was detected for Fas, FasL, and TRAIL; adult mouse liver and spleen mRNAs were used as controls (Fig. 2A). IL-6 analyses are presented elsewhere (Melnick et al., 2000).

As shown in Figure 2B, there was a 4-fold increase ($P < 0.01$) of TNF mRNA from E14 to E18, i.e., from mostly *Pseudoglandular Stage* to mostly late *Terminal Bud Stage*. Similarly, there was a 1.3-fold increase ($P < 0.01$) in TNF-R1 transcript and a nearly 2-fold increase ($P < 0.01$) in Caspase 8 mRNA. There were virtually no changes from E14 to E18 for TRADD or RIP transcript levels.

Of particular note was the highly significant correlation between TNF, TNF-R1, and Caspase 8 mRNA levels (Table 1). As correlation does not necessarily reflect causal relationship, and as each is highly correlated with increasing gestational age (Table 1), a correlation between the two is very likely. In fact, analysis of covariance (ANCOVA) reveals that Caspase 8 varies very significantly ($P < 0.001$) with gestational age even when TNF and TNF-R1 levels are held constant, suggesting the important role of additional pathways in the regulation of Caspase 8 expression with progressive SMG morphogenesis.

Protein immunohistochemical analysis. Previous studies have demonstrated the spatial and temporal pattern of TNF, TNF-R1 and TNF-R2 immunolocalization in embryonic SMG branching epithelia (Jaskoll and Melnick, 1999), with TNF being diffusely distributed throughout the branching epithelia and TNF receptors exhibiting a more restricted spatial distribution associated with forming ductal and terminal bud lumina. Specifically, TNF receptors are seen on the ductal epithelial surfaces facing the forming lumina, at sites of ductal lumen formation,

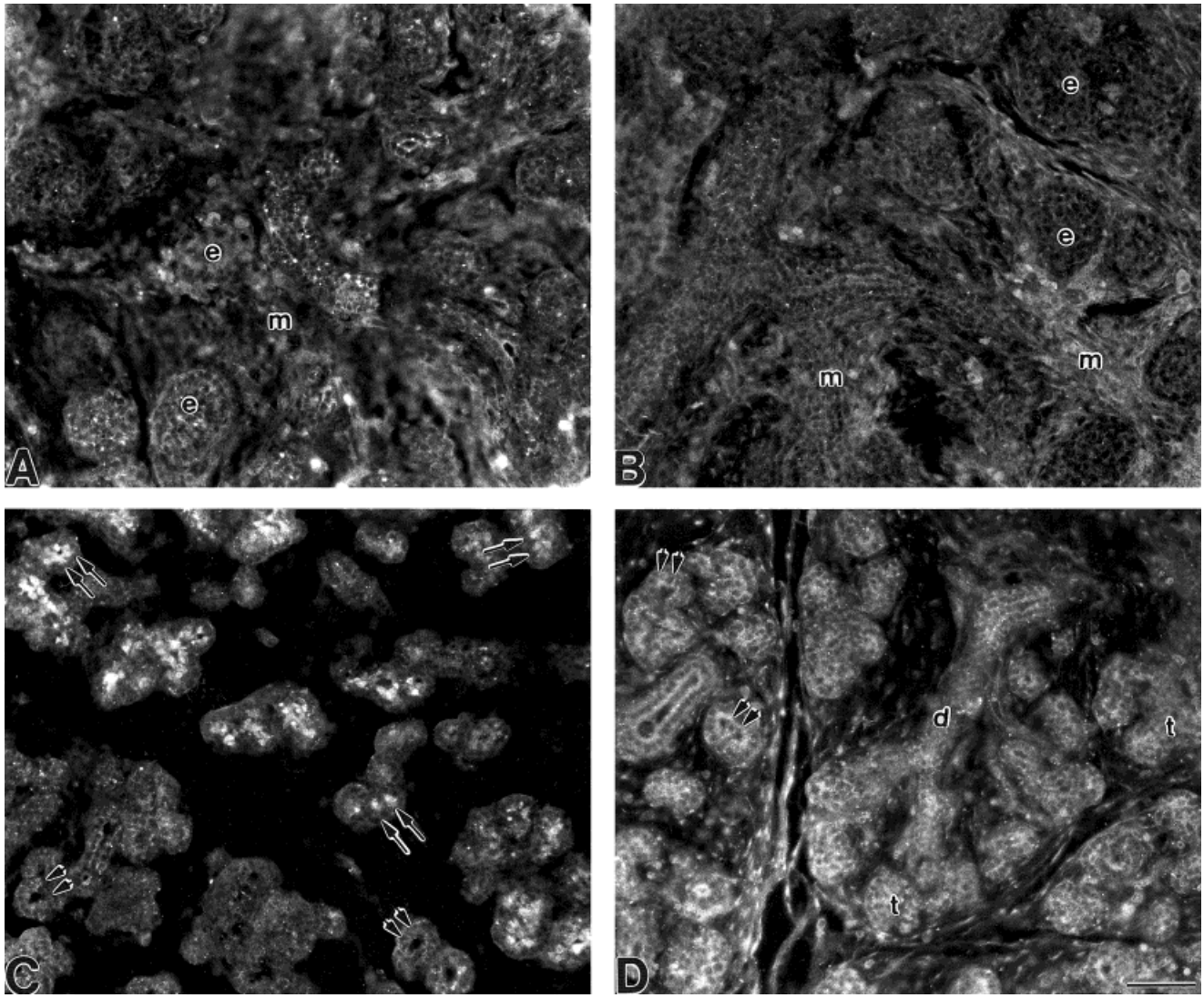


Fig. 3. Immunolocalization of NFκB and IκB during embryonic SMG development. **A,B:** The *Pseudoglandular Stage.* **C,D:** The *Canalicular* and *Early Terminal Bud Stages*. Note that both the Canalicular and Early Terminal Bud Stages of SMG development are seen in a single histologic section. In the *Pseudoglandular Stage*, NFκB (A) is primarily immunolocalized in branching epithelia (e), and to a lesser extend in the mesenchyme (m). By contrast, IκB (B) is diffusely distributed primarily in the mesenchyme, and to a lesser extent, in the epithelia during this stage. By the *Canalicular Stage*, NFκB (C) is primarily immunodetected in the central region of terminal end buds (double arrows), and to a lesser extent, at sites of ductal lumen formation (data not shown). By contrast, IκB (D) is diffusely distributed throughout ductal (d) and terminal bud (t) epithelia. By the *Early Terminal Bud Stage* in which lumina are present in some terminal buds (double arrowheads), both NFκB (C) and IκB (D) are both diffusely distributed throughout ductal and terminal bud epithelia. NFκB exhibits less intense immunostain, however, than that seen for IκB. Scale bar = 50 μm.

and in the central regions of the terminal buds that will later exhibit lumen formation. Because TNF binding to TNF-R1 can potentially transduce either a cell proliferation or apoptosis signal, we investigated downstream components of the two different TNF signal transduction pathways. NFκB is the transcription factor known to transduce the proliferation signal as well as to inhibit the apoptosis signal. In the *Pseudoglandular Stage*, NFκB is primarily immunodetected in SMG branching epithelia, and, to a much lesser extent, in the mesenchyme (Fig. 3A). By the *Canalicular Stage*, NFκB is primarily immunolocalized in the central regions of the terminal buds, and to a lesser extent, on the ductal surfaces facing the lumina (Fig. 3C); this pattern is similar to that seen for TNF-R1 (Jaskoll and Melnick, 1999). By the *Terminal Bud Stage*, NFκB is diffusely distributed throughout ductal and terminal bud epithelia (Fig. 3C), with the intensity of immunostain being markedly diminished compared with the *Canalicular Stage*. Given that (1) NFκB is normally found in unstimulated cells in an inactive cytoplasmic form bound to IκB, its inhibitory protein; and (2) TNF induction of IκB phosphorylation releases NFκB for translocation to the nucleus to bind to NFκB response genes (Fig. 1), we also investigated the spatiotemporal distribution of IκB

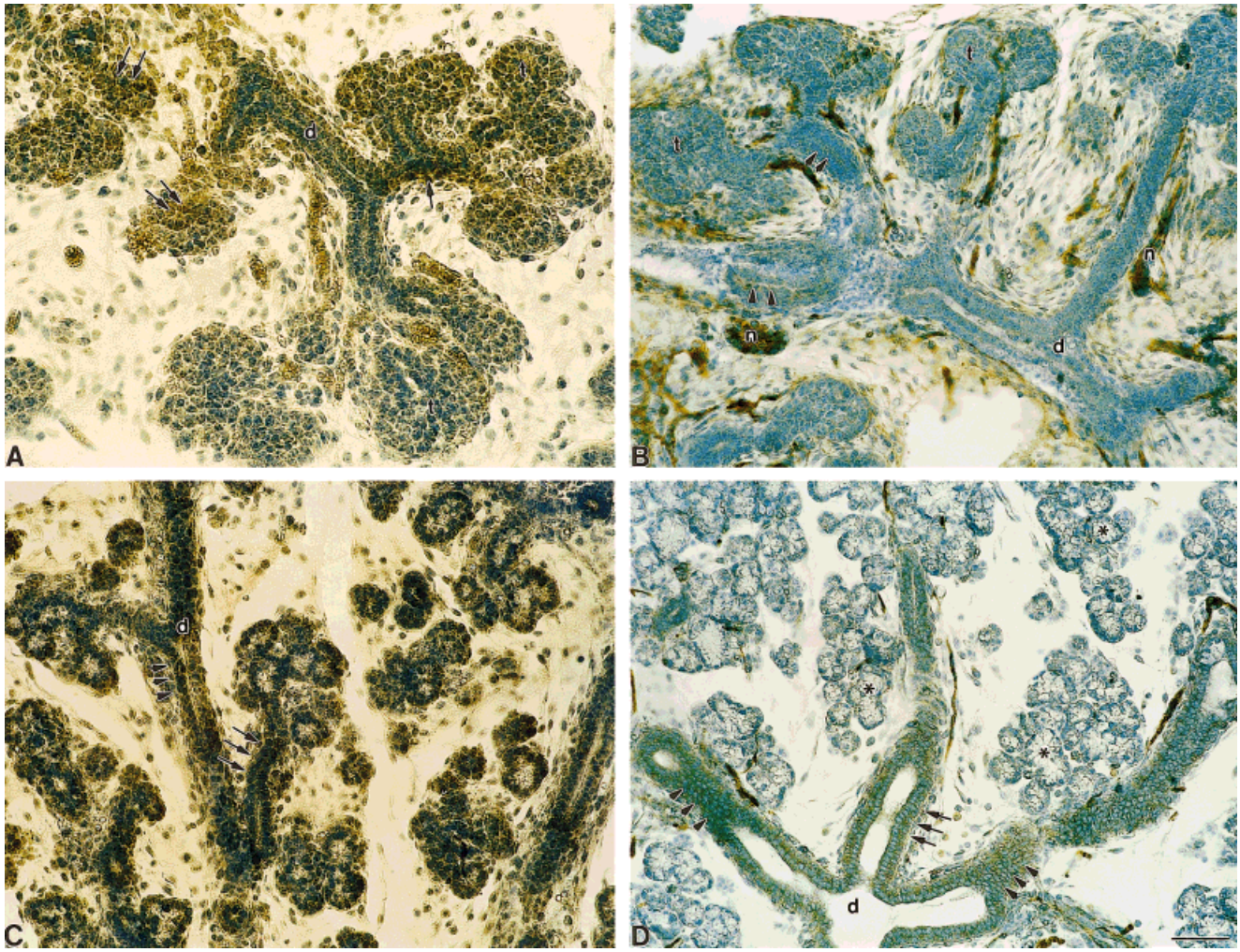


Fig. 4. Immunolocalization of total Caspase 8 and activated Caspase 3 in embryonic SMGs. **A,B:** *Canalicular Stage.* **C,D:** *Early Terminal Bud Stage*. In the early *Canalicular* SMG, Caspase 8 protein (A) is immunolocalized throughout ductal (d) and terminal end bud (t) epithelia; more intense immunostain is seen in ductal epithelia (arrow) and terminal end bud central regions (double arrows) at sites that will later exhibit lumena. At this same stage of development, activated Caspase 3 (B) is less apparent, being immunodetected in ductal epithelium at sites of lumen formation (double arrowheads). At the later stage of development, total Caspase 8 (C) and activated Caspase 3 (D) are colocalized in ductal epithelia surrounding lumena (triple arrows) and at sites that will later exhibit lumena (triple arrow heads). Caspase 8 protein is also detected in terminal end bud epithelia whereas activated Caspase 3 is absent (*). Note the high level of activated Caspase 3 immunostain in nerves (n). Scale bar = A,B 75 μm; C,D 50 μm.

relative to that seen for NFκB (Fig. 3) . IκB immunolocalization markedly differs from that seen for NFκB during the *Pseudoglandular* and *Canalicular Stages* (compare Figs 3B,D to 3A,C). That is, IκB is diffusely distributed primarily throughout the mesenchyme in the *Pseudoglandular Stage* (Fig. 3B) and throughout ductal and terminal end bud epithelial in both the *Canalicular* and *Terminal Bud Stages* (Fig. 3D).

TNF/TNF-R1 transduces its apoptotic signal through the subsequent activation of a cascade of caspase enzymes, specifically the activation of initiator Caspase 8 which, in turn, activates effector Caspase 3 (Fig. 1). Accordingly, we determined the spatial distribution of Caspase 8 and activated Caspase 3 (Fig. 4). Because antibody is unavailable to specifically detect activated Caspase 8, our studies demonstrate the presence of total Caspase 8, both precursor and activated protein. Nevertheless, because an antibody is available to identify activated Caspase 3, and pro-Caspase 3 activation is dependent *in part* on the presence of activated Caspase 8, employing these two antibodies and identifying sites of colocalization enables one to deduce cells likely to be coexpressing both activated Caspase 8 and activated Caspase 3. In the *Early Canalicular* SMG, Caspase 8 protein is present throughout ductal and terminal bud epithelia, with more intense immunostain being seen in ductal epithelia surrounding forming lumina and terminal bud central regions that will later exhibit lumina (Fig. 4A). By contrast, activated Caspase 3 is very weakly immunodetected in early *Canalicular* SMG epithelia, primarily in the ductal epithelium at sites of lumen formation (Fig. 4B). The relative absence of immunodetectable

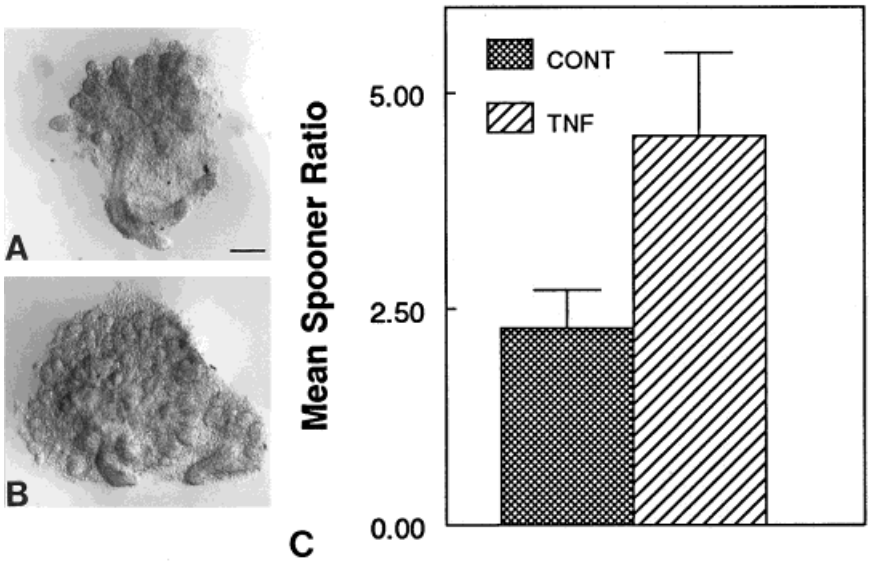


Fig. 5. Effect of TNF on early embryonic SMG branching morphogenesis in vitro. **A:** E13 (*Pseudoglandular Stage*) SMG primordium cultured for 3 days in control medium. **B:** E13 SMG primordium cultured in the presence of 10 U/ml TNF for 3 days. TNF supplementation substantially increases branch number compared with control. **C:** Mean ± SEM Spooner ratios (72 hr/24 hr) of control (n = 6) and TNF-treated (n = 7) explants. Arithmetic ratios were log transformed for analysis (see Experimental Procedures). TNF treatment induces a significant increase in branching morphogenesis ($P < 0.04$).

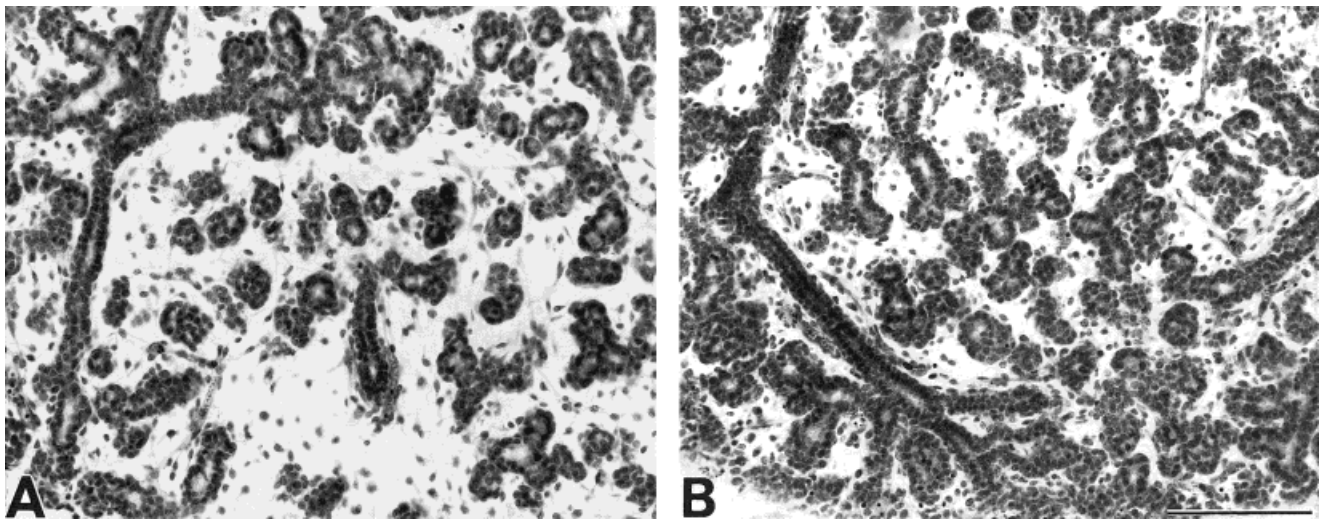


Fig. 6. Effect of TNF supplementation on *Canalicular Stage* SMGs in vitro. **A:** E15 primordium cultured in control medium for 4 days (E15 + 4). **B:** E15 primordium cultured in the presence of 10 U/ml TNF for 4 days (E15 + 4). TNF supplementation induces a substantial increase in ductal and terminal bud branches. Scale bar = 100 μm.

activated Caspase 3 suggests that little, if any, TNF-mediated apoptosis is occurring at this stage of development. With progressive development, Caspase 8 (Fig. 4C) and activated Caspase 3 (Fig. 4D) are colocalized to ductal epithelia surrounding lumina and at sites that will later exhibit lumina. However, although Caspase 8 protein is immunolocalized in terminal bud epithelia, the absence of immunodetectable activated Caspase 3 in terminal buds suggests that TNF/TNF-R1 modulated apoptosis primarily mediates SMG ductal canalization but not terminal bud lumen formation.

Effect of TNF on Embryonic SMG Morphogenesis In Vitro

The presence of TNF signal transduction pathway components is suggestive but not probative of TNF regulation of embryonic SMG development. To address this question, we cultured early embryonic (E13) SMGs in the presence or absence of TNF supplementation for 3 days and evaluated branching morphogenesis as previously described (Jaskoll et al., 1994b). TNF supplementation induces a significant 2-fold increase ($t_{11} = 2.43$; $P < 0.04$) in SMG

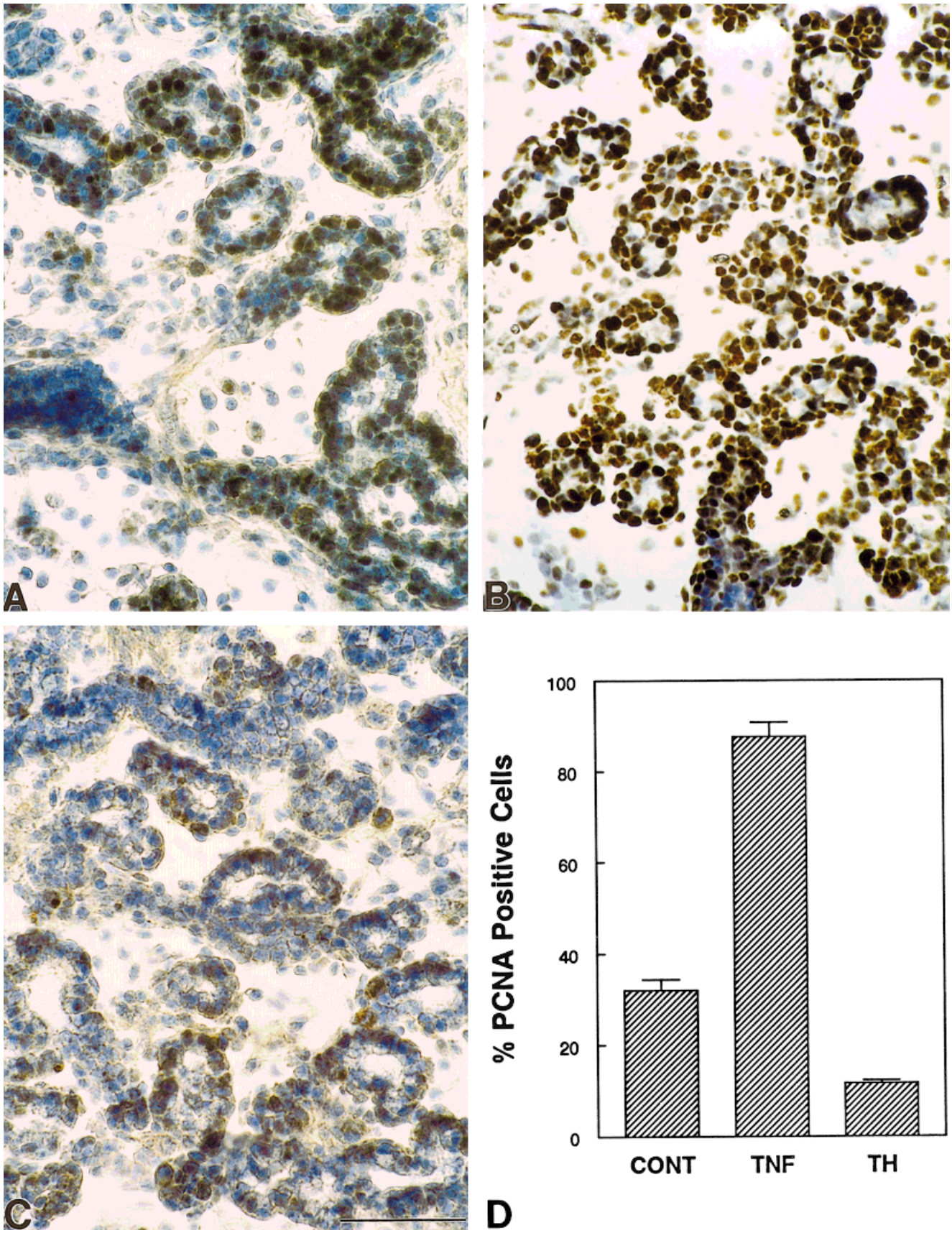


Fig. 7. Embryonic SMG cell proliferation in vitro. **A:** E15 + 4 control explant. **B:** E15 + 4 TNF-treated explant. **C:** E15 + 4 TH-treated explant. Cell proliferation (black color) is markedly increased with TNF treatment (B) compared with control (A). TH treatment (C) results in a substantial decrease in cell proliferation compared with control (A). Scale bar = 50 μm. **D:** Mean ± SEM percent PCNA positive epithelium in control, TNF-treated, and TH-treated explants. TNF-treated is nearly 3-fold greater than control ($P < 0.001$); TH-treated is almost ⅔ less than control ($P < 0.001$). Percents were arcsin transformed for analysis (see Experimental Procedures). Each bar is the mean of three independent samples; each independent sample represents counts in three randomly selected regions of that sample.

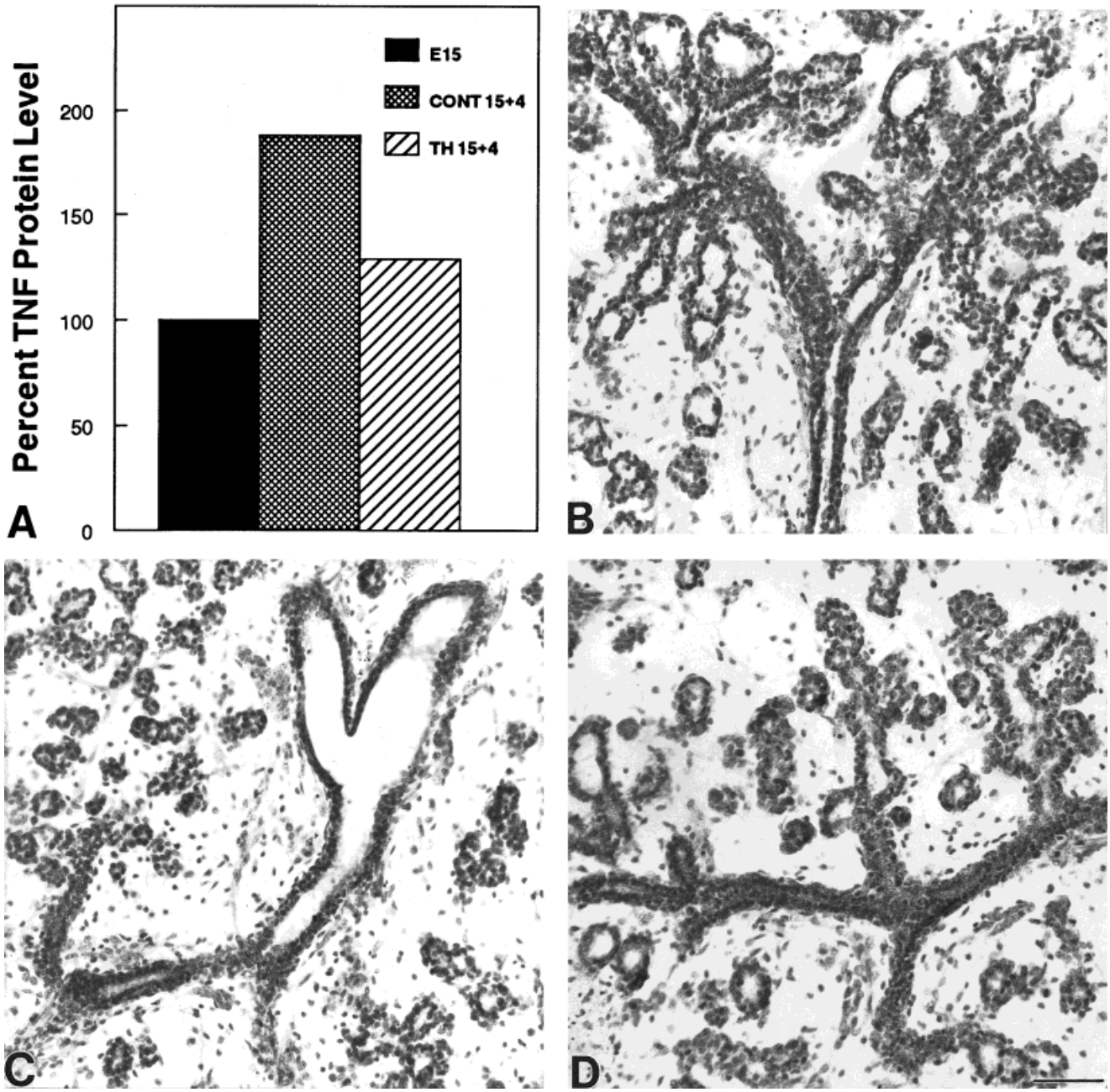
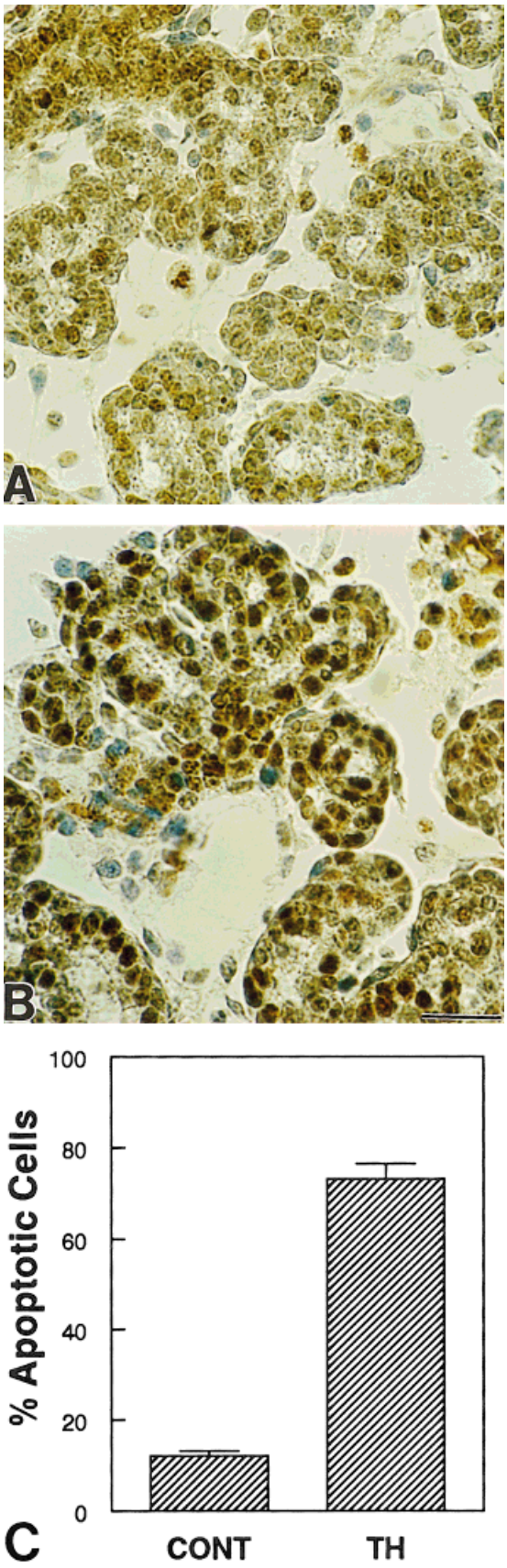


Fig. 8. **A:** Endogenous TNF protein level in developing embryonic SMGs. The level of TNF protein in control and TH-treated E15 SMG primordia cultured for 4 days is presented as a percent of TNF protein level in E15 starting tissue; n = 18 for each bar. Control explants exhibit a nearly 2-fold increase in endogenous TNF protein level compared with E15 primordia. TH treatment results in ⅔ less endogenous TNF protein than in control explants. This difference is highly significant ($P < 0.001$). **B–D:** Histological evaluation of control (B), TH-treated (C), and TH + TNF-treated (D) E15 + 4 primordia. TH treatment markedly decreases epithelial branching as well as enlarging ductal lumen size (*); the explants are rescued by exogenous TNF protein. Scale bar = 50 μm.

branch number in vitro (Fig. 5). Because prior immunolocalization studies suggest that TNF signal transduction primarily occurs during later stages of embryonic SMG development (*Canalicular* and *Terminal Bud*) (Jaskoll and Melnick, 1999), we also evaluated the affect of exogenous TNF on E15 (mostly *Canalicular*) SMG primordia in vitro after 4 days in culture. Again, there is a substantial increase in ductal branching and number of terminal buds in TNF-treated explants compared with controls (compare Fig. 6A to 6B). Further, we quantitated PCNA-defined epithelial cell proliferation in the presence or absence of TNF supplementation (Fig. 7). TNF treatment in vitro induces a highly significant 3-fold increase ($t_4 = 11.20$; $P < 0.001$) in epithelial cell proliferation as com-



pared with controls (Fig. 7A,B,D). TNF supplementation clearly enhances embryonic SMG morphogenesis in vitro.

Because previous studies have demonstrated that TNF induces branching morphogenesis and histodifferentiation in pulmonary and mammary gland explants in vitro (Ip et al., 1992; Jaskoll et al., 1994a), we determined if TNF supplementation induces acinar-specific mucin expression in cultured embryonic SMGs. E15 SMG explants were cultured for 4 days (E15 + 4) or 7 days (E15 + 7) in the presence or absence of TNF supplementation, and possible TNF-induced differences in mucin protein were determined by immunohistochemistry. No differences were seen between control and TNF-supplemented explants on Days 4 or 7 of culture (data not shown), indicating that TNF does not induce embryonic SMG mucin protein expression.

Inhibition of the TNF Pathway In Vitro

Supplementation experiments with exogenous TNF are not necessarily dispositive for the role of *endogenous* TNF during embryonic SMG development. Accordingly, we employed an inhibitory experimental strategy to subvert the TNF pathway. Cultured explants we treated with thalidomide (TH), a drug well documented to decrease endogenous TNF levels by enhancing TNF mRNA degradation (Moreira et al., 1993; Lopez-Talavera et al., 1996). Dose-response studies determined that 20 μg/ml was the most effective dose (data not shown). On Day 4 of culture, control explants exhibit an approximate 2-fold increase in endogenous TNF protein compared with E15 starting primordia (Fig. 8A), indicating that embryonic SMG primordia synthesize endogenous TNF protein in vitro. Although endogenous TNF protein is present in TH-treated explants, the level is only 1.3-fold greater than in E15 starting primordia, or ⅔ less than the increase in control (E15 + 4) explants (Fig. 8A). This highly significant (t_∞ = 3.92; $P < 0.001$) TH-induced decline in endogenous TNF protein production results in a highly significant ⅔ decline (t_4 = 9.51; $P < 0.001$) in SMG epithelial cell proliferation (Fig. 7C,D) and branching morphogenesis (Fig. 8B,C) compared with controls. TH-treated explants could be rescued with exogenous TNF supplementation: when E15 SMG primordia were cultured in the presence of TH and TNF, their epithelial cell proliferation and branching morphogenesis resembled that seen in control explants (Fig. 8D). It should be noted that the phenotypic outcomes of explant exposure to TNF and TH, respectively, are polar opposites (Fig. 7B–D). Additionally, the TH-induced decline in endogenous TNF protein expression results in a significant 6-fold increase (t_4 = 16.25; $P < 0.001$) in apoptosis (Fig. 9A–C). Few apoptotic cells were seen in TNF rescued TH-treated explants, with the relative level being similar to that seen in controls (data not shown). Our

Fig. 9. Thalidomide induces a substantial increase in apoptosis. **A:** E15 + 4 control explants. **B:** E15 + 4 TH-treated explants. There is a marked increase in apoptotic cells (brown color) with TH treatment (B) compared with control (A). Scale bar = 25 μm. **C:** Quantitative differences in apoptosis between control and TH-treated explants: Mean ± SEM percent apoptosis positive epithelium in control and TH-treated explants; TH-treated is 6-fold greater than control ($P < 0.001$); each bar is the mean of three independent samples, each representing three randomly selected regions of that sample.

observation that TNF supplementation can rescue TH-treated explants indicates that the TH phenotype is due to TNF insufficiency rather than TH toxicity.

DISCUSSION

The proper balance between cellular proliferation, quiescence, and apoptosis must be maintained to sustain normal organogenesis. An imbalance between these processes can result in organ aplasia, hypoplasia, hyperplasia, or dysplasia. The cells of a developing organ are, in an anthropomorphic sense, altruistic. They survive and differentiate when needed and are suicidal when not. The latter seems to be the default state; sufficient apoptosis-suppressing signals potentiate the former (Raff, 1998). In studying the ontogeny of any organ, then, the key is understanding how and what signals are initiated and integrated to achieve morphogenetic homeostasis. Regarding submandibular salivary gland (SMG) development, our present study indicates that TNF/TNF-R1 signaling is important in this regard.

In vivo analysis of the developing SMG revealed the presence of TNF, TNF-R1, TRADD, RIP, and Caspase 8 mRNA transcripts, but not the presence of Fas, FasL, and TRAIL mRNA. TNF, Fas, and TRAIL pathways share the same cytoplasmic facilitator molecules (e.g., TRADD, FADD, TRAF) and the same dual functions of caspase-mediated apoptosis and NFκB-mediated cell survival and proliferation (Ashkenazi and Dixit, 1998; Gravestein and Borst, 1998; Jeremias and Debatin, 1998; Wallach et al., 1999). Absence of Fas, FasL, and TRAIL mRNA suggests tissue specificity for TNF/TNF-R1 superfamily member function. It is possible that these transcripts are present at low, undetectable levels, awaiting immediate recruitment should the TNF/TNF-R1 signal be insufficient. This possibility is supported by the presence of FAF (Fas-associated factor) transcript (Fig. 2); FAF protein binds to the intracellular portion of Fas and potentiates apoptosis (Chu et al., 1995; Frölich et al., 1998). Significant increases of FAF transcript with gestational age ($P < 0.001$) also suggest the possibility that the Fas pathway is important to postnatal SMG development. In this regard, there was also a highly significant increase of TNF, TNF-R1 and Caspase 8 with increasing gestational age (SMG maturation), but not so for TRADD and RIP that remained constant. Because TRADD and RIP are rate-limiting in the TNF signal cascade, this may simply reflect variation in mRNA stability that can differ by several orders of magnitude in higher eukaryotic cells (Sachs, 1993).

In vivo analysis of relevant proteins revealed immunodetectable TNF, TNF-R1, TNF-R2, IκB, NFκB, Caspase 8, and activated Caspase 3. TNF, TNF-R1, TNF-R2, IκB, NFκB, and Caspase 8 were localized to both ductal and terminal bud epithelia. Activated Caspase 3, by contrast, was only present in ductal epithelia. This suggests that presumptive acini formation is not Caspase 3-dependent. Other studies in our laboratory suggest that it is p53-dependent (Melnick and Jaskoll, 2000).

TNF is a pleiotropic cytokine that modulates growth through the TNF-R1/NFκB cascade and apoptosis through the TNF-R1/Caspase 8 cascade (Fig. 1). To be sure, the mere presence of TNF, TNF-R1, and its associated proteins in developing SMGs, even if pattern specific, does not provide prima facie evidence of TNFs role in SMG morphogenesis. As a first approach to this question we utilized a serumless, chemically defined SMG organ culture model to investigate the effect of TNF supplementation on embryonic SMG primordia. The optimum TNF dose induced a 3-fold increase in cell proliferation and a 2-fold increase in epithelial branching. Although TNF is known to induce pulmonary and mammary gland histodifferentiation in vitro as indicated by the up-regulation of surfactant-associated proteins and casein, respectively (Ip et al., 1992; Jaskoll et al., 1994a), we report here that TNF supplementation did not up-regulate acinar-cell specific mucin protein expression. This result is not unexpected because embryonic SMG mucin has been shown to notably differ from that seen in the adult gland (Jaskoll et al., 1998) and is not a SMG terminal differentiation product. In this regard, embryonic SMG mucin is not analogous to either surfactant or casein.

Using the same organ culture model, we induced TNF insufficiency with thalidomide (TH). The phenotypic outcome was the polar opposite of that with TNF supplementation. The optimum TH dose reduced endogenous TNF level by ⅔ and SMG cell proliferation by nearly⅔. Additionally, TH-induced TNF insufficiency results in a significant increase in epithelial apoptosis. In vitro, alternative (redundant) signal transduction pathways do not seem adequate to rescue TNF insufficiency. These results with TH are similar to those seen with suppression of NFκB activity (Kolenko et al., 1999).

The TNF-R1/NFκB cascade is the primary signal transduction pathway induced by TNF ligand binding and receptor trimerization (Ashkenazi and Dixit, 1998). Among the many activators of NFκB, the most potent are TNF and IL-1 (Karin, 1998). Activated NFκB translocates to the nucleus and binds cognate DNA to enhance cell proliferation and inhibit apoptosis (Fig. 1) (Perkins, 1997; Karin, 1998). These dual effects on cell life and death have been termed the "Janus faces of NFκB" (Lipton, 1997), a reference to the Roman guardian of portals with two faces looking in opposite directions. NFκB enhances proliferation by stimulating the expression of cytokines such as TNF, IL-1, IL-2, IL-6, and IL-8, among others (Perkins, 1997); NFκB inhibits apoptosis by inducing TRAF and cIAP protein expression that suppresses Caspase 8 activation (Wang et al., 1998), and by inhibition of p53 gene expression (Webster and Perkins, 1999). Notably, studies of TNF and TNF-R1 null mice indicate that other pathways may compensate for absent TNF/TNF-R1 signaling (Rothe et al., 1993; Pasparakis et al., 1996; Marino et al., 1997; Peschon et al., 1998). This is hardly surprising because it is now known that there are over 150 inducers of NFκB and over 150 target genes (Pahl, 1999). The challenge of TNF or TNF-R1 gene deletion and loss of function, then, enhances alternative NFκB inductive pathways that are otherwise functionally minor.

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