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Interleukin-6 Signaling and Embryonic Mouse Submandibular Salivary Gland Morphogenesis

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Key Words

Submandibular salivary gland · Interleukin-6 · Tumor necrosis factor · gp130 · STAT3 · bcl-2 · Mucin · Development · Mouse

Abstract

Interleukin-6 (IL-6) is a multifunctional cytokine that mediates cell growth, differentiation, and survival. It was the objective of the present study to investigate the possible function(s) of IL-6 signaling 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 strategies to investigate the phenotypic outcomes of enhanced IL-6-induced signaling and immunoperturbation of IL-6 binding to cognate receptors. These experiments demonstrate: (1) there is a significant increase of IL-6 mRNA with progressive SMG development, and that this is highly correlated with TNF transcript levels; (2) IL-6 and its cognate receptors are immunolocalized in SMG branching epithelia from the canalicular stage to the late terminal bud stage, as are other constituents of the IL-6 pathway; (3) as compared to controls, IL-6-supplemented explants exhibit a substantial increase in overall size and in the number of ductal branches and terminal buds, as well as a highly significant increase in epithelial cell proliferation; (4) SMG explants cultured in the presence of anti-IL-6 neutralizing antibodies exhibit a marked decrease in epithelial ducts and terminal buds, concomitant with a significant decline in cell proliferation and a highly significant increase in apoptosis. Taken together, our experimental results indicate that IL-6 signaling is important to SMG developmental homeostasis.

Abbreviations used in this paper

GREs	glucocorticoid response elements
IgG	immunoglobulin G
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
JAK	Janus kinase
MAPK	mitogen-activated protein kinase
NFκB	nuclear factor κB
PCNA	proliferating cell nuclear antigen
P-STAT3	phosphorylated STAT3
RAS	a family of oncogenes that encode GTPases
SMG	submandibular gland
SHP	a tyrosine phosphatase which contains SH2 domains
STAT3	signal transducer and activator of transcription-3
TNF	tumor necrosis factor

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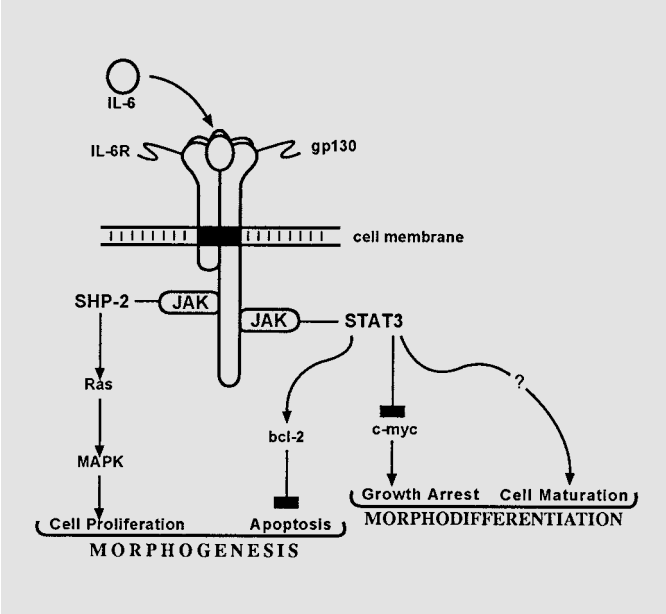


Fig. 1. IL-6/IL-6R/gp130 signal transduction pathway. Based on reviews by Heinrich et al. [1998] and Hirano [1998].

Introduction

Mouse embryonic submandibular gland (SMG) organogenesis [Wessells, 1977; Jaskoll and Melnick, 1999] begins about embryonic day (E) 11.5 as a single epithelial stalk with a single lobule or bud at its distal end. This SMG primordium branches by repeated furcation at the distal ends of successive buds to produce a bush-like structure comprised of a network of elongated epithelial branches and terminal epithelial buds. These branches and buds hollow out to form the ductal system and presumptive acini, respectively. At any given gestational age, beginning around E14, one will find more than one stage of development represented in the embryonic organ. Nevertheless, at any given gestational age there will be a preponderance of one stage (pseudoglandular, canalicular, or terminal bud) relative to others. Normal progressive SMG development is maintained by properly balanced cell proliferation, quiescence, and apoptosis. This developmental homeostasis is mediated by hormones, growth factors, and cytokines [see review, Melnick and Jaskoll, 2000]. One such cytokine which is expressed in the canalicular and terminal bud stages of SMG organogenesis is interleukin-6 (IL-6) [Jaskoll and Melnick, 1999].

IL-6 is a multifunctional cytokine that mediates cell growth, differentiation, and survival [see reviews by Heinrich et al., 1998; Hirano, 1998]. IL-6 is a glycoprotein with a molecular mass ranging from 21 to 28 kD. IL-6 expression is regulated by a variety of factors, including up-regulation by tumor necrosis factor (TNF) and IL-1 via the nuclear factor κB (NFκB) pathway and down-regulation by glucocorticoid (CORT) via the CORT/CORT receptor (GR) pathway. Correspondingly, cis-regulating elements in the 5′ flanking region of the IL-6 gene include glucocorticoid response elements (GREs) and NFκB binding sites. The binding of IL-6 to its cognate transmembrane receptor (IL-6R) induces the formation of a hexamer composed of two each of IL-6, IL-6R, and gp130, a 130-kD transmembrane signal transducer. Hexamer formation induces a gp130 specific signaling cascade (fig. 1): tyrosine phosphorylation of constitutively associated Janus tyrosine kinases (JAKs) → activation of signal transducer and activator of transcription 3 (STAT3) and/or the SHP2/Ras/MAPK mitogenic pathway. STAT3 activation enhances growth arrest and differentiation. gp130-induced cell growth utilizes two signals, SHP2/Ras/MAPK mitogenic signals and an anti-apoptotic signal mediated by STAT3 via up-regulation of bcl-2. While it appears that IL-6 can promote both cell proliferation signals and growth arrest/differentiation signals simultaneously, the former is most apparent when STAT3 activation is suppressed. Thus, which IL-6 induced signaling pathways are activated in a given cell at a given time and place depends on the expression pattern of a whole range of signaling molecules.

It was the objective of the present study to investigate the possible function(s) of IL-6 signaling in SMG morphogenesis. After characterizing in vivo mRNA and protein expression of various constituents of this complex pathway, we utilized in vitro strategies to investigate the phenotypic outcomes of enhanced IL-6 induced signaling and immunoperturbation of IL-6 binding to cognate receptors. The results of the experiments indicate that IL-6 signaling is important to SMG developmental homeostasis.

Materials and Methods

Tissue Collection

Female B10A/SnSg mice, obtained from Jackson Laboratories (Bar Harbor, Me., USA), were maintained and mated as previously described [Jaskoll et al., 1994]; plug day = day 0 of gestation. Pregnant females were anesthetized on days 15–18 of gestation (E15–18) with methoxyflurane (metafane) and euthanized by cervical dislocation. Embryos were dissected in cold phosphate buffered saline (PBS) and staged according to Theiler [1989]. SMGs were dissected and processed for histology as described below or stored at –70°C.

Culture System

E15 SMG primordia were cultured using a modified Trowell method as previously described [Jaskoll et al., 1994]. The medium consisted of BGJb (Life Technologies, Rockville, Md., USA) supplemented with 0.5 mg ascorbic acid/ml and 50 units penicillin/streptomycin (Life Technologies), pH 7.2; medium was changed every other day. Cultures were supplemented on day 0 with different concentrations of recombinanant mouse IL-6 (0.05, 0.5, or 5 ng/ml rIL-6) (R&D Systems, Minneapolis, Minn., USA) and maintained for 4 days; the concentrations used were 2–100 times the concentration previously shown to induce cell proliferation in the IL-6 dependent plasmacytoma cell line, T1165.85.2.1. After determining that 5 ng/ml elicited a marked response, we then conducted 3 independent experiments consisting of a minimum of 8 E15 SMG primordia cultured in control medium or 5 ng/ml rIL-6 supplementation for 4 days. Tissue was evaluated at time of collection and processed for morphologic or cell proliferation analyses as described below.

Interruption Studies. IL-6 signal transduction was interrupted using anti-mouse IL-6 neutralizing antibodies (R&D Systems). The concentrations used (0.05, 0.5 and 1.0 μg/ml) were 2–100 times the concentration previously shown to suppress IL-6 induction (R&D Systems); identical concentrations of goat IgG (R&D Systems) were used as a control to evaluate nonspecific effects. SMG morphogenesis was evaluated in a minimum of 6 explants per group. After determining that 0.5 or 1.0 μg/ml anti-IL-6 antibodies elicited a similar marked response in embryonic SMGs, 0.5 μg/ml was then used in all subsequent studies. Three independent experiments were conducted, consisting of a minimum of 8 CONT, 8 CONT-IgG and 8 anti-IL-6-treated explants per experiment. SMG morphogenesis was evaluated in a minimum of 5 explants per experimental group on day 4 or 7 of culture by routine light microscopy. To delineate the pathogenesis of decreased branching by anti-IL-6 antibodies, E15 primordia were cultured for 2 days (E15 + 2) in control medium, anti-IL-6 antibodies, or goat IgG; 3 independent experiments were conducted, each group consisting of a minimum of 8 explants per group. SMG morphogenesis, cell proliferation or apoptosis was evaluated in a minimum of 3 explants per experimental group.

Histology and Immunochemistry

SMGs were fixed in Carnoy's fixative, processed, embedded in low-melting point paraplast, and immunostained as previously described [Jaskoll and Melnick, 1999]. In all experiments, negative controls were incubated in the absence of primary antibody or with preimmune serum; controls were routinely negative. A minimum of 4 SMGs were evaluated for each stage of development or per experimental group.

Antibodies: Polyclonal antibodies to IL-6, IL-6R, gp130, phosphorylated STAT3 and bcl2 were purchased from Santa Cruz Biotech, Inc (Santa Cruz, Calif., USA). Polyclonal antibodies to SMG mouse mucin were prepared in our laboratory as previously reported [Jaskoll et al., 1998].

Cultured explant morphogenesis was evaluated by light microscopy of serial sections stained with hematoxylin and eosin. For all experimental groups, a minimum of 5 explants per group was evaluated.

Proliferation Assay

Sections were incubated with anti-PCNA using the Zymed (South San Francisco) mouse PCNA kit and then counterstained with hematoxylin. For cell proliferation quantitation, 3 independent sections per group were selected and 3 areas per section were photographed at ×200. PCNA-positive epithelial cells/total epithelial cells were determined per area and the mean ratio PCNA-positive epithelial cells per section was determined.

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, Fla., USA) as previously described [Jaskoll and Melnick, 1999]. In contrast to the TUNNEL method, monoclonal antibodies to ssDNA have been shown to be specific for apoptotic cell death and do not detect necrotic cells. 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) prior to 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.

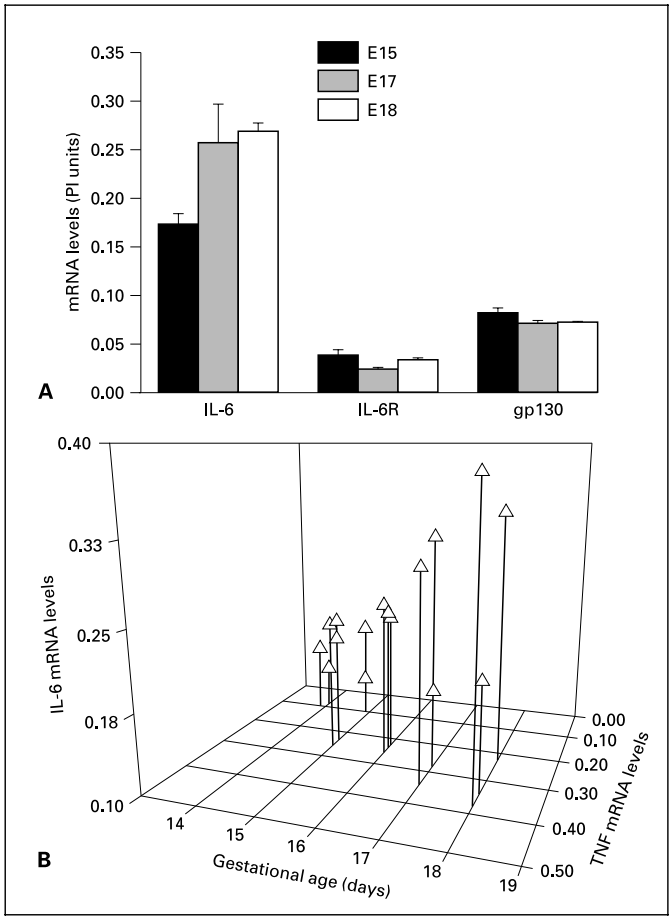
Apoptosis was evaluated in a minimum of 4 explants per experimental group. Quantitation of apoptosis was conducted as described above for cell proliferation. Apoptosis is presented as the ratio of apoptotic-positive epithelial cells/total epithelial cells. Mean ratios per section and mean ratios per group were determined.

mRNA Quantitation

Total RNA was isolated from a minimum of 3 litters of E14, E15, E17, and E18 SMGs as previously described [Jaskoll et al., 1998]. RNAse protection assay was conducted using Riboquant Multiprobe kits (Pharmingen, San Diego, Calif., USA) to evaluate IL-6, IL-6R, gp130, and TNF; 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% polyacrylamice, 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 gestational day.

Statistical Analysis

Means and variances were analyzed utilizing t-test, 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, ratios and percentages were log or arcsin transformed [Sokal and Rohlf, 1981].



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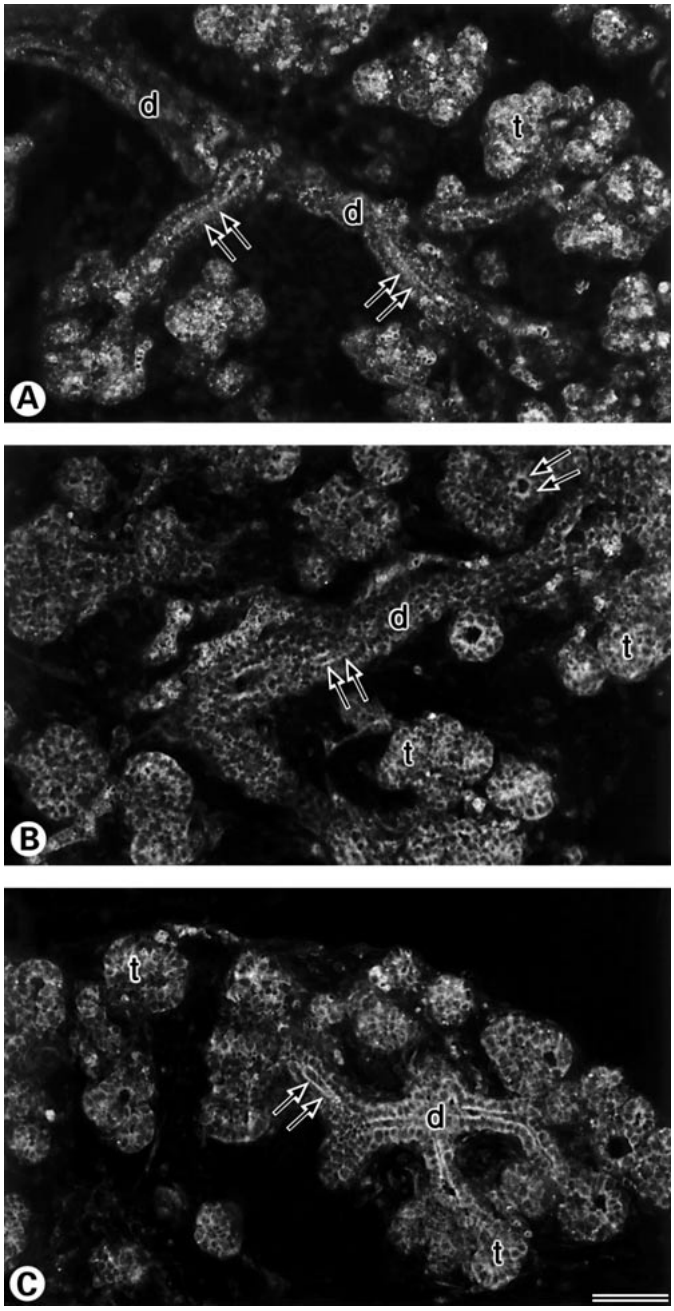

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Fig. 2. mRNA quantitative analysis. **A** Quantitative analysis for 3 relevant transcripts during embryonic SMG development. Bar graph of means ± SEMs for 3 relevant transcription products in E15, E17, and E18 SMGs; n = 3 for each bar. A significant increase from E15 to E18 was found for IL-6. **B** Covariation of IL-6, TNF, and gestational age ('day').

Fig. 3. Immunolocalization of the IL-6 signal transduction pathway constituents in developing SMGs. A. IL-6. B. IL-6R. C. GP130. IL-6 is primarily localized in epithelia surrounding forming ductal (d) lumina (arrows) and throughout terminal bud (t) epithelia. IL-6R and gp130 are colocalized on ductal and terminal end bud epithelia membranes; more intense immunostain is seen on epithelial surfaces facing forming lumina (arrows). Bar = 50 μm.

Results

Developmental Expression of the IL-6 Signal Transduction Pathway

mRNA Quantitative Analysis. To investigate the expression in developing SMGs of genes relevant to IL-6 signaling, we used a method that permitted us to measure mRNA levels for multiple genes simultaneously in each independent sample (Methods). These included IL-6, IL-6R, gp130, and TNF. The choice of IL-6, IL-6R, and gp130 is obvious since they are common to both the JAK/STAT3 and the SHP2/Ras/MAPK cascades (fig. 1). TNF was measured because TNF is present during SMG duct and presumptive acini formation [Jaskoll and Melnick, 1999] and is a major inducer of IL-6 expression via downstream NFκB DNA binding [Hirano, 1998]. Quantitation

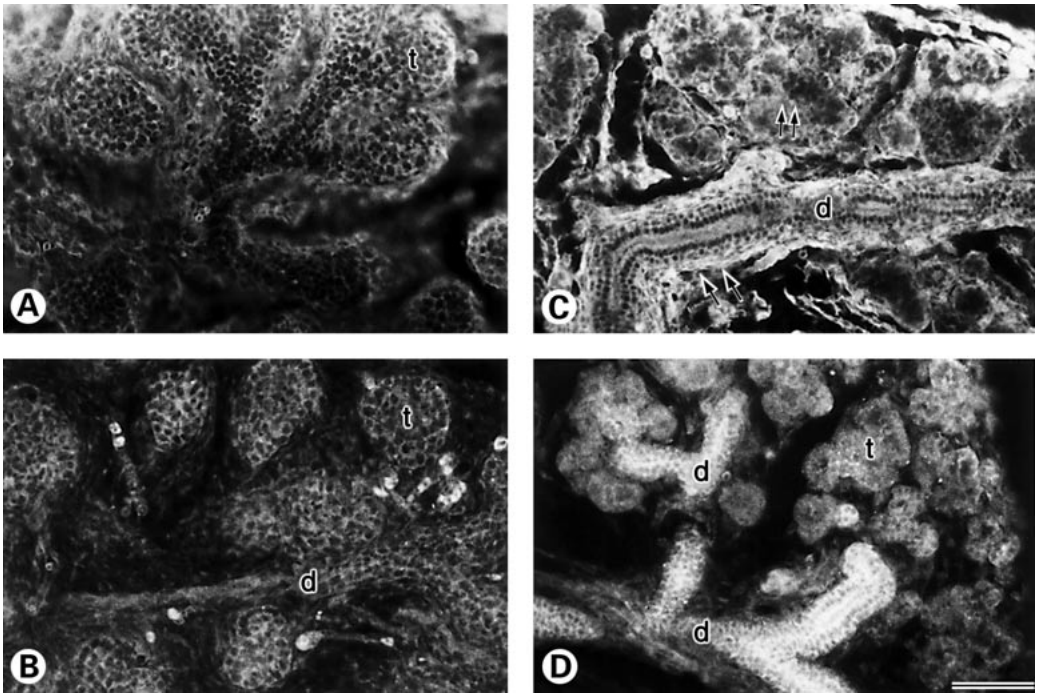

Fig. 4. Immunolocalization of P-STAT3 and bcl-2 during embryonic SMG development. **A**, **B** Canalicular stage. P-STAT3 (**A**) is mostly localized in terminal bud (t) epithelia and bcl-2 (**B**) is localized in ductal (d) and terminal end bud epithelia. **C**, **D** Terminal bud stage. P-STAT3 (**C**) is localized in ductal epithelia, as well as in ductal and terminal bud mesenchyme (arrows). bcl-2 (**D**) is localized in ductal and terminal bud epithelia. Bar = 50 μm.

of mRNA was made on 3 independent samples for each gestational age.

As shown in figure 2A, there was a 1.5-fold increase ($p < 0.01$) of IL-6 mRNA transcript from mostly canalicular stage to mostly terminal bud stage. There were virtually no changes of IL-6R and gp130 mRNA transcript levels. Of particular note is the highly significant positive correlation ($r = 0.69$; $p < 0.01$) between TNF and IL-6 mRNA levels. Since correlation does not necessarily reflect causation, each could be correlated with one another because each is highly correlated with increasing gestational age (fig. 2B). However, analysis of covariance (ANCOVA) reveals that IL-6 does not differ significantly ($p > 0.25$) among days when TNF mRNA levels are held constant. In fact, nearly half the variation of IL-6 mRNA levels can be accounted for by the variation in TNF mRNA levels ($r^2 = 0.48$). Of course, half the variation is associated with as yet unidentified factors.

Protein Immunohistochemical Analysis. IL-6, IL-6R, and gp130 are immunolocalized in embryonic SMG branching epithelia from the canalicular stage to the late terminal bud stage (fig. 3). IL-6 is primarily immunolocalized in epithelia surrounding forming ductal lumina, as well as throughout terminal bud epithelia (fig. 3A). IL-6R (fig. 3B) and gp130 (fig. 3C) are colocalized on ductal and terminal bud epithelia membranes, with more intense immunostain visualized on epithelial surfaces facing forming lumina.

IL-6 induced gp130 signal transduction can initiate one of several pathways (fig. 1); STAT3 and bcl-2 expression can suggest which one(s) [Hirano, 1998]. To this end, we determined the spatiotemporal distribution of phosphorylated (activated) STAT3 (P-STAT3) and bcl-2 protein relative to that seen for IL-6, IL-6R, and gp130. In early canalicular stage SMGs, P-STAT3 is detected throughout terminal bud epithelia and, to a lesser extent, in ductal epithelia and mesenchyme (fig. 4A); bcl-2 is also localized throughout ductal and terminal bud epithelia (fig. 4B). In late canalicular and terminal bud stages, P-STAT3 is largely immunolocalized to ductal epithelia and surrounding mesenchyme, only occasionally seen in terminal bud epithelia (fig. 4C); bcl-2 remains strongly de-

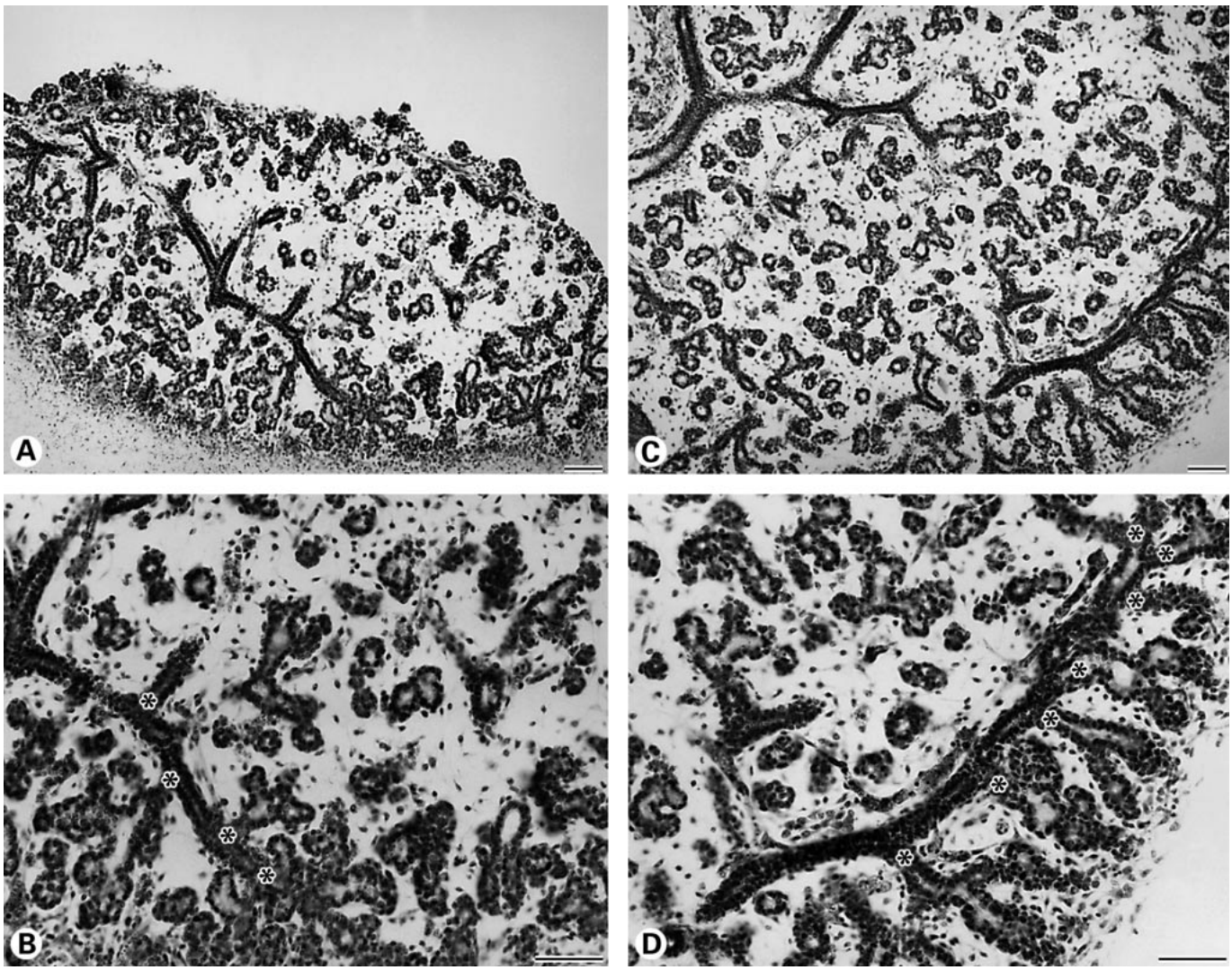

Fig. 5. Effect of IL-6 supplementation on canalicular stage SMGs in vitro. **A**, **B** E15 primordium cultured in control medium for 4 days (E15 + 4). **C**, **D** E15 primordium cultured in the presence of 5 ng/ml IL-6 for 4 days (E15 + 4). Exogenous IL-6 supplementation induces a substantial increase in SMG overall size and in the number of terminal buds compared to controls. Note that IL-6 (**D**) induces a marked increase in terminal bud branch points (*) from the primary duct compared to that seen in control (**C**). Bar = 50 μm.

tected in both ductal and terminal bud epithelia (fig. 4D). Taken together, the data in figures 3 and 4 are consistent with IL-6 induction of the morphogenesis pathway (fig. 1) [Hirano, 1998], namely cell proliferation and suppression of apoptosis. Also, P-STAT3 localization in mesenchyme in the absence of IL-6 and its cognate receptors, indicates the presence of other, unidentified activators of STAT3.

Effect of IL-6 on Embryonic SMG Morphogenesis in vitro

The presence of IL-6 signal transduction pathway components is suggestive but not probative of IL-6 regulation of embryonic SMG development. Since our immunolocalization studies (fig. 3, 4) suggest that IL-6 signaling primarily occurs during later stages of SMG development (canalicular and terminal bud), we addressed this question by culturing E15 (mostly canalicular) SMG primordia in the presence or absence of IL-6 supplementation for

4 days. Dose-response studies determined that 5 ng/ml was the most effective dose to elicit a response on day 4 of culture (data not shown). IL-6-supplemented explants exhibit a substantial increase in overall size and in the number of ductal branches and terminal buds as compared to controls (compare fig. 5A, B to 5C, D). Further, we quantitated PCNA-defined epithelial cell proliferation in the presence or absence of IL-6 supplementation (fig. 6). IL-6 treatment in vitro induces a highly significant 3-fold increase ($p < 0.001$) in epithelial cell proliferation as compared to controls. IL-6 supplementation clearly enhances embryonic SMG morphogenesis in vitro.

Interruption of IL-6 Signaling in vitro

Since supplementation experiments with exogenous IL-6 are not necessarily dispositive for the role of endogenous IL-6 during embryonic SMG development, we employed an immunoperturbation strategy to subvert IL-6 signaling. Investigators have utilized a variety of IL-6 neutralization strategies in vitro and in vivo, including anti-IL-6 antibody, soluble IL-6R, anti-IL-6R antibody, and IL-6R antagonists [Rettig, 1997; Jourdan et al., 1999]. We chose to interrupt IL-6 signaling in vitro using neutralizing antibody to IL-6. Dose-response studies determined that 0.5–1.0 μg/ml anti-IL-6 antibodies elicited a response in E15 SMG primordia cultured from 2 to 7 days (data not shown). Controls consisted of culture medium alone or supplementation with 0.5–1.0 μg/ml goat IgG; IgG-supplemented control SMG primordia are phenotypically similar to those cultured in control medium alone (data not shown). SMG primordia cultured in the presence of anti-IL-6 neutralizing antibody exhibit a marked decrease in epithelial ducts and terminal buds compared to control explants, with substantially larger ductal lumina being seen in treated explants (compare fig. 7A, B to 7C, D).

Given that IL-6 signaling induces both Ras/MAPK mediated cell proliferation and bcl-2 mediated suppression of apoptosis (fig. 1) [Hirano, 1998], we questioned

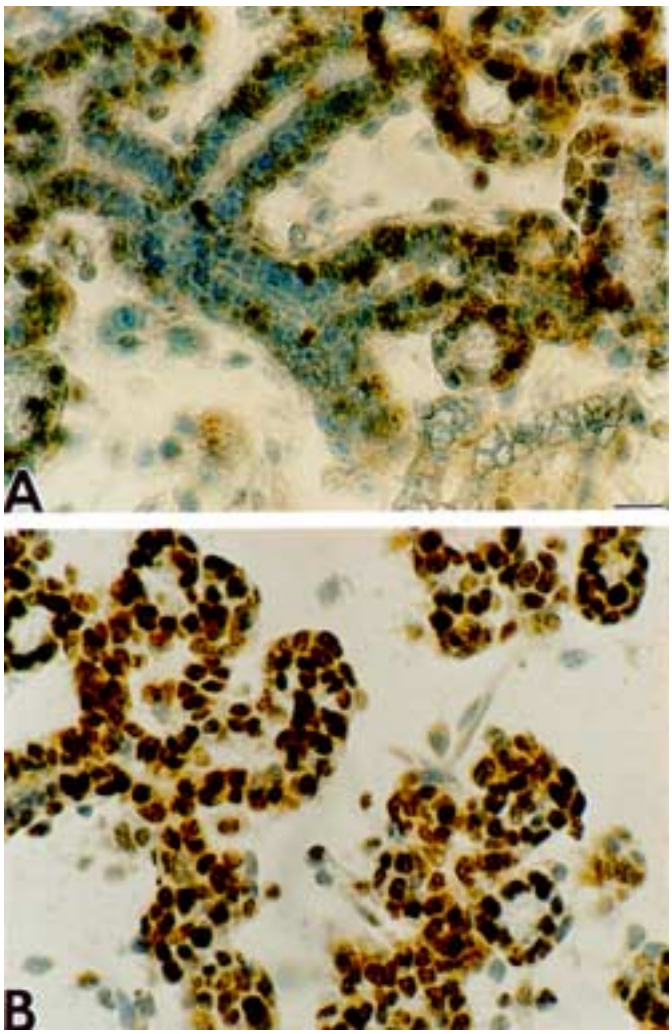

Fig. 6. IL-6 supplementation induces SMG cell proliferation in vitro. **A** E15 + 4 control explant. **B** E15 + 4 IL-6 supplemented explant. A substantial increase in PCNA-positive cells (brown color) is seen in the presence of IL-6 supplementation compared to control. Bar = 50 μm. **C** Quantitation of PCNA-positive cells demonstrates that IL-6 supplementation induces a significant 3-fold increase ($p < 0.001$) in cell proliferation. The data is presented as mean percent PCNA-positive cells ± SEM.

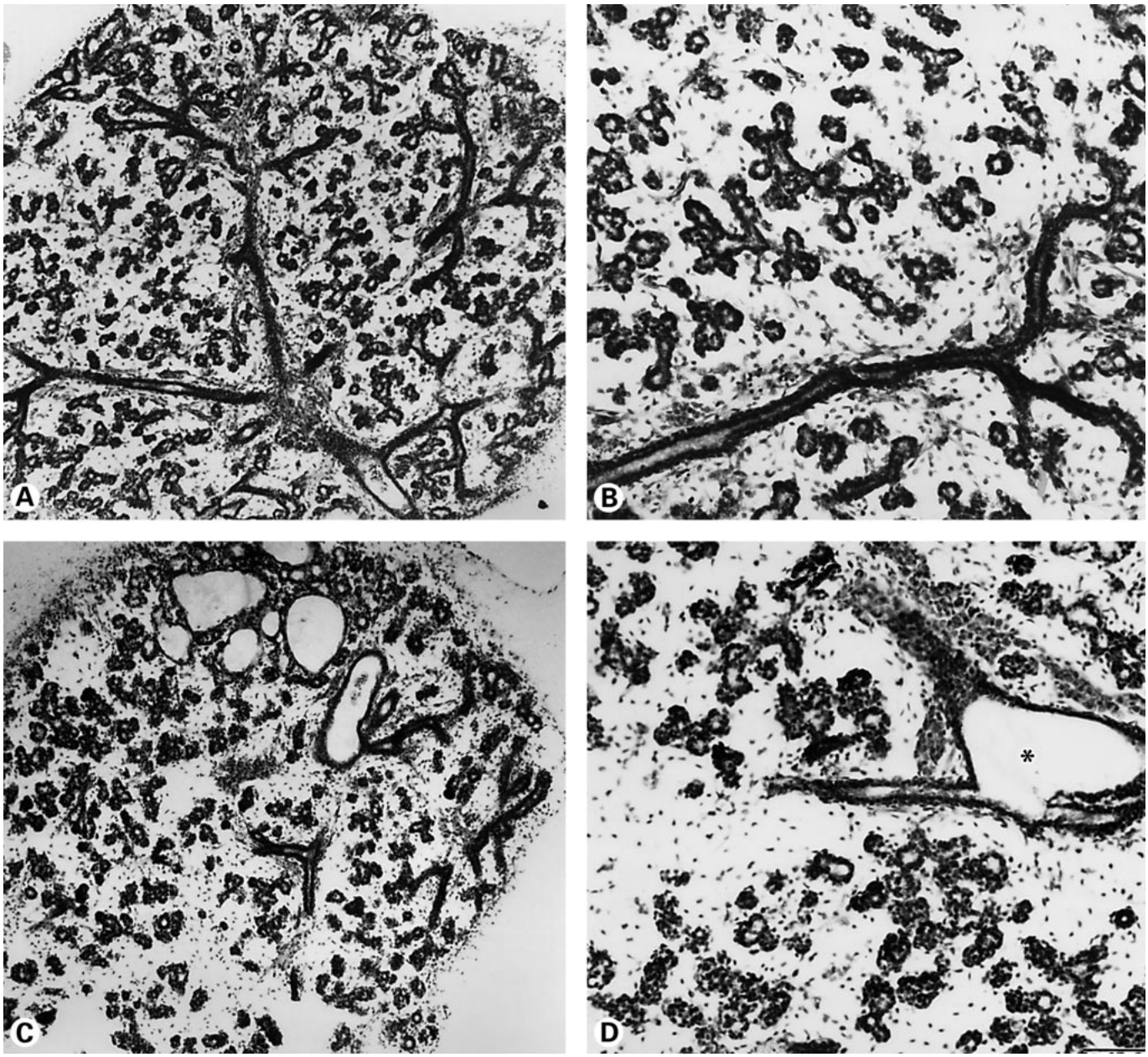

Fig. 7. Anti-IL-6 antibodies interrupt embryonic SMG development in vitro. **A**, **B** Control 15 + 4 SMG. **C**, **D** Anti-IL-6 treated 15 + 4 SMG. Immunoperturbed IL-6 signaling results in smaller explants exhibiting a substantial decrease in ducts and terminal buds compared to controls. Note the abnormally large ductal lumina (*) in treated explants. Bar = **A**, **B** 100 μm; **C**, **D** 50 μm.

whether the observed decline in epithelial branching in anti-IL-6-treated explants is due to decreased cell proliferation, increased apoptosis, or both. E15 SMG primordia cultured for 2 days demonstrate a highly significant 60% decline ($p < 0.001$) in cell proliferation (fig. 8A, B, 9A) and a highly significant 13-fold increase ($p < 0.001$) in apoptosis (fig. 8C, D, 9B). Concomitant with the latter, there is a marked absence of immunodetectable P-STAT3 and bcl-2 protein in the epithelium of IL-6 antibody treated explants compared to controls (fig. 10).

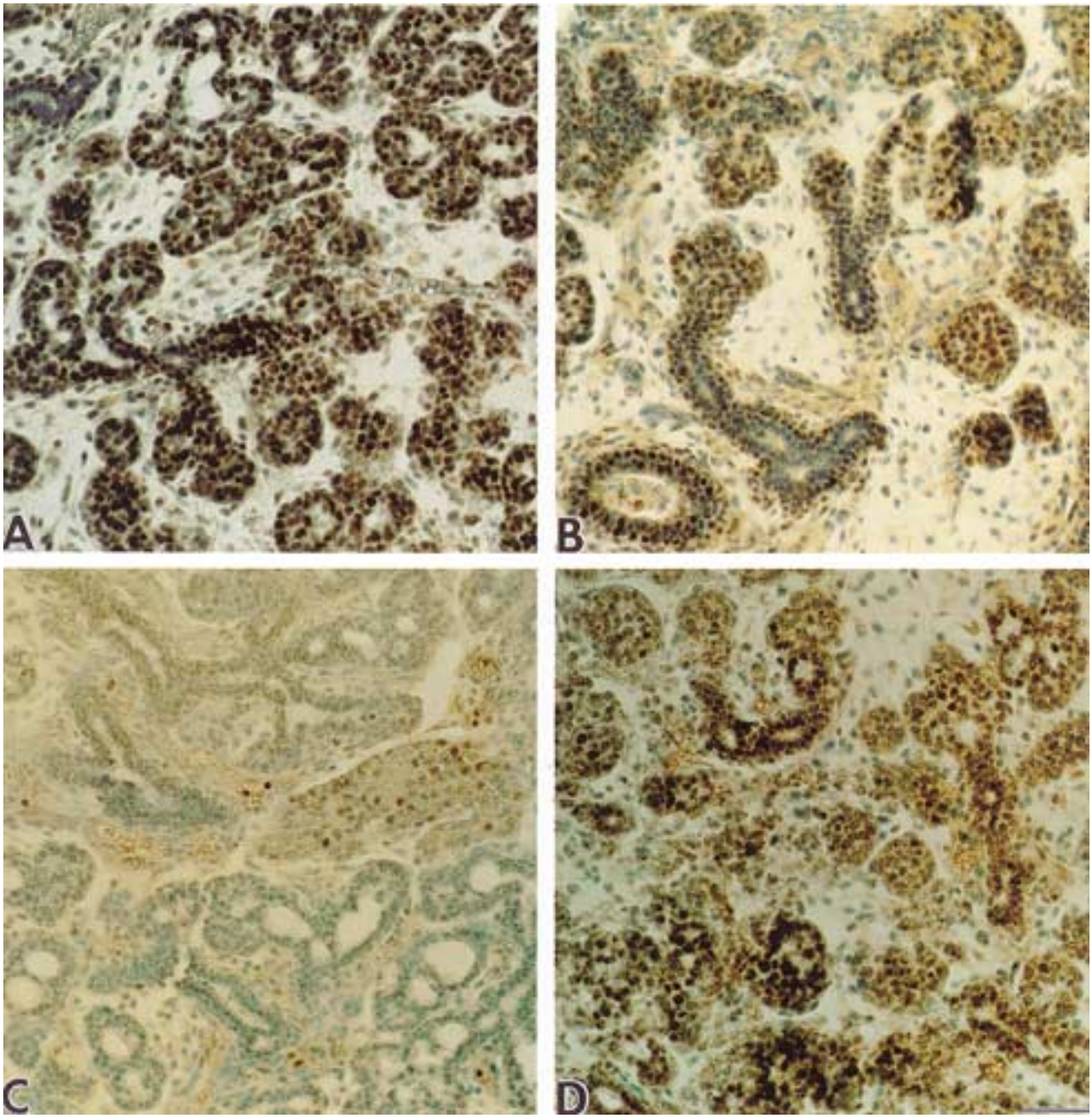

Fig. 8. Anti-IL-6 antibodies suppress SMG cell proliferation and induce apoptosis in vitro. **A**, **B** Cell proliferation. PCNA-positive cells (brown color) in control (**A**) and anti-IL-6 immunopertubated (**B**) E15 SMG primordia cultured for 2 days. There is a substantial decrease of PCNA-positive cells in treated explants compared to control. **C**, **D** Apoptosis. Apoptotic-positive cells (brown color) in control (**C**) and anti-IL-6-treated (**D**) E15 primordia cultured for 2 days. With anti-IL-6 treatment, a substantial increase in apoptotic-positive cells is seen compared to control. Bar = 50 μm.

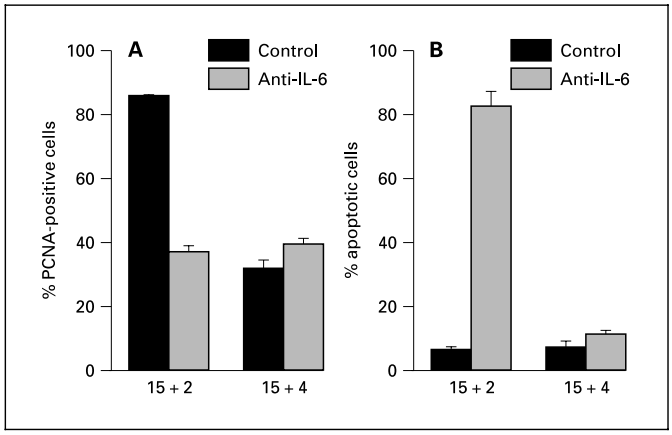

Fig. 9. Quantitation of PCNA-positive cells and apoptotic cells on day 2 (15 + 2) and day 4 (15 + 4) of culture. **A** Percent PCNA-positive cells. A significant decline ($p < 0.001$) in cell proliferation is seen in treated SMGs on day 2; cell proliferation is similar in control and treated 15 + 4 SMGs. The data presented is mean percent PCNA-positive cells ± SEM. **B** Percent apoptotic-positive cells. A significant increase ($p < 0.001$) in apoptosis is seen with immunopertubation on day 2 of culture; apoptosis is similar in control and treated 15 + 4 SMGs. The data presented is mean percent apoptotic cells ± SEM.

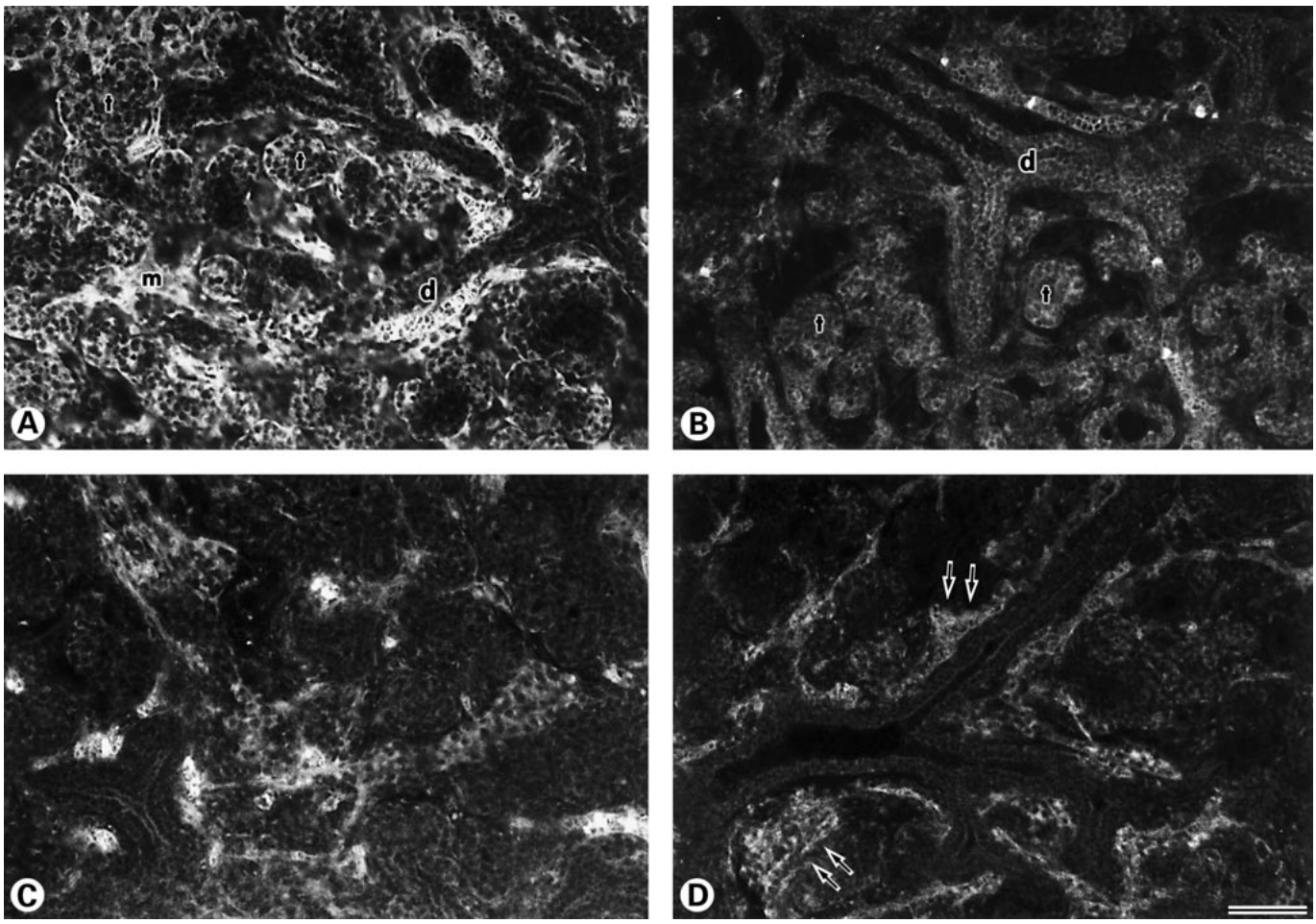

Fig. 10. Anti-IL-6 antibodies suppress immunodetectable P-STAT3 and bcl-2 in vitro. **A**, **B** Control E15 + 2 explants. **C**, **D** E15 + 2 explants cultured in the presence of anti-IL-6 antibody. In control explants, P-STAT3 (**A**) is seen in ductal (d) and terminal bud (t) epithelia, as well as in surrounding mesenchyme (m). bcl-2 (**B**) is localized throughout ductal and terminal end bud epithelia and is absent from mesenchyme. With treatment, there is near absence of immunodetectable P-STAT3 (**C**) and bcl-2 (**D**) in ductal and terminal bud epithelia compared to controls. In addition, treated explants also exhibit an marked increase in immunodetectable bcl-2 in the mesenchyme (arrows). Bar = 50 μm.

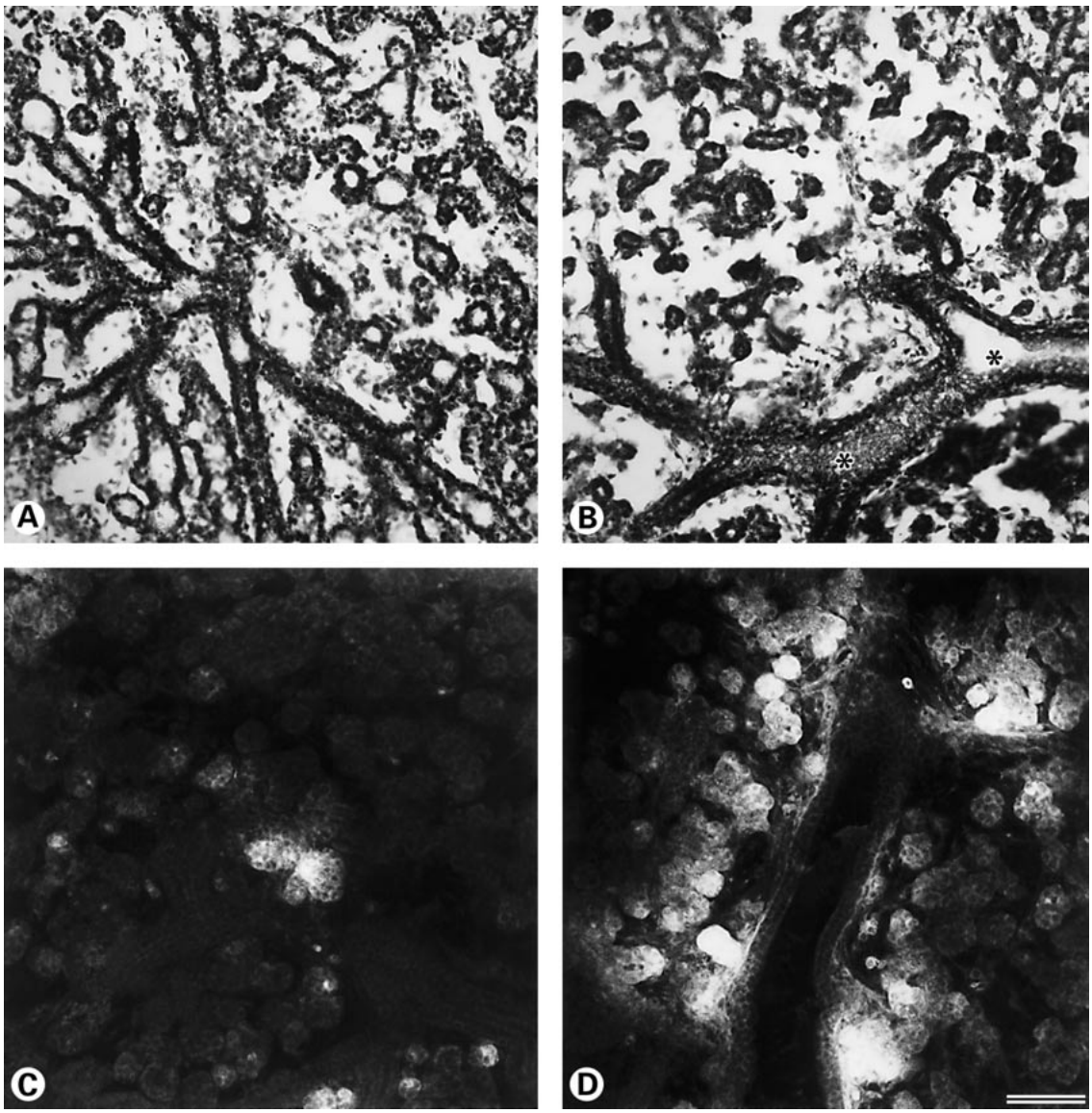

Fig. 11. Effect of anti-IL-6 antibodies on E15 SMG primordia cultured for 7 days. **A**, **C** Control E15 + 7 SMGs. **B**, **D** E15 + 7 SMGs cultured with anti-IL-6 antibodies. **A**, **B** SMG morphology: Anti-IL-6 antibodies (**B**) induce a marked decrease in the number of ducts and terminal buds compared to control (**A**); anti-IL-6 treatment also results in a marked increase in the size of ductal lumena (*). **C**, **D** Embryonic mucin protein expression: a substantial increase in immunodetectable embryonic mucin protein is seen in anti-IL-6-treated explants (**D**) compared to control (**C**). Bar = 50 μm.

It is interesting to note that after 4 days of culture there are no longer appreciable differences in either cell proliferation (fig. 9A) or apoptosis (fig. 9B) between IL-6 antibody treated and control. This suggests 'recovery' via an alternate pathway, a proposition supported by our studies of embryonic mucin expression, a marker of terminal bud maturation [Jaskoll et al., 1998]. We cultured E15 SMG primordia in the presence or absence of anti-IL-6 neutralizing antibody for 7 days, the minimum time period for the detection of embryonic mucin protein in control explants [Jaskoll et al., 1998]. On day 7 of culture, IL-6 antibody-treated explants exhibit fewer terminal buds and ducts (with larger lumina) compared to controls (compare fig. 11A, B); there is a substantial increase in

immunodetectable embryonic mucin with IL-6 antibody treatment (compare fig. 11C, D). Upregulation of embryonic mucin expression, then, occurs in the absence of IL-6 signaling and P-STAT3 expression, a known mediator of cell maturation (fig. 1) [Heinrich et al., 1998; Hirano, 1998], again suggesting an alternate development pathway in IL-6 antibody-treated explants.

Discussion

Organogenesis is largely dependent upon developmental homeostasis, i.e. the proper balance between cellular proliferation, quiescence, and apoptosis. The cells of a developing organ survive and differentiate when needed and are suicidal when not. The latter appears to be the default state; sufficient apoptosis-suppressing signals potentiate the former [Raff, 1998]. Regarding submandibular salivary gland (SMG) development, our present study indicates that IL-6 has a significant role in maintaining morphogenetic homeostasis.

IL-6 is a multifunctional cytokine that transduces its signal by binding to its cognate receptors, IL-6R and gp130. It is via gp130 that IL-6 activates multiple intracellular signaling pathways (fig. 1): (1) activation of the Ras/MAPK mitogenic pathway occurs in parallel with activation of the JAK-STAT3 differentiation pathway [Raz et al., 1999]; (2) morphogenesis depends on STAT3-regulated, bcl-2 anti-apoptosis [Heinrich et al., 1998; Rollwagen et al., 1998; Gottlieb, 1999] and on SHP2-mediated mitotic signaling [Fukada et al., 1996; Heinrich et al., 1998]; (3) morphodifferentiation depends on STAT3-mediated signals for growth arrest and differentiation [Yamanaka et al., 1996], as well as anti-apoptosis signals [Bellido et al., 1998; Hirano, 1998]; (4) since there are reciprocal roles for STAT3- and SHP2-mediated signals [Ohtani et al., 2000], activated STAT3 levels appear to be a key to the cellular choice between morphogenesis and morphodifferentiation, low levels favoring the former and higher levels the latter [Nakajima et al., 1996; Hirano, 1998].

In vivo analysis of the developing SMG revealed the presence of IL-6, IL-6R, and gp130 mRNA transcripts. While IL-6 significantly increases with gestational age, IL-6R and gp130 do not. It was found that nearly half the variation in IL-6 transcript levels could be accounted for by the variation in TNF transcript levels, independent of gestational age. This is consistent with many prior studies which show that TNF upregulates IL-6 mRNA levels [Katz et al., 1994; Kurokouchi et al., 1998]. In vivo analysis of relevant IL-6 signaling pathway proteins revealed immunodetectable IL-6, IL-6R, gp130, activated STAT3 and bcl-2 in ductal and terminal bud epithelia. It is noteworthy that activated STAT3 appears to be expressed at low levels and bcl-2 is expressed at high levels in canalicular stage and terminal bud stage epithelia, a time when there is a high degree of cell proliferation and a low degree of apoptosis [Jaskoll and Melnick, 1999]. This is consistent with IL-6-induced morphogenesis rather than morphodifferentiation [Fukada et al., 1996; Heinrich et al., 1998; Hirano, 1998].

Nevertheless, the mere presence of IL-6 and its associated proteins in developing SMGs, even if pattern specific, does not provide prima facie evidence of IL-6's 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 IL-6 supplementation on embryonic SMG primordia. The optimum IL-6 dose induced a substantial increase in the number of ductal branches and terminal buds, as well as a 3-fold increase in PCNA-defined epithelial cell proliferation.

Using the same organ culture model, we applied an often used IL-6 neutralization strategy, anti-IL-6 neutralizing antibody [Rettig, 1997; Jourdan et al., 1999]. After 2 days in culture, the optimum antibody dose induced a notable decline in the number of ductal branches and terminal buds, a 2/3 decline in PCNA-defined epithelial cell proliferation, and a 13-fold increase in apoptosis. Concomitant with this was the marked absence of immunodetectable activated STAT3 and bcl-2 protein in ductal and terminal bud epithelium.

Taken together, our experimental results with IL-6 supplementation and IL-6 neutralization indicate that IL-6 plays an important role regarding induction of cell proliferation and suppression of apoptosis in the epithelia of developing SMGs in utero. It remains to be determined if IL-6 signaling is important to growth arrest and terminal histodifferentiation.

Finally, it is interesting to note that after 4 days of culture IL-6 neutralization no longer induced any appreciable differences in cell proliferation or apoptosis, as compared to control. Further, IL-6 neutralized 7-day explants showed an increase of immunodetectable mucin protein. This unexpected IL-6 neutralized phenotype suggests 'recovery' via alternate pathways. In this regard, several points are informative. First, IL-6 knockout mice show normal embryogenesis while knockouts of gp130, JAK, STAT3, and SHP2 are embryolethal [Heinrich et al., 1998]. Second, TNF can function as a cell survival and

proliferation factor in the absence of IL-6 signaling [Jourdan et al., 1999], probably because NFκB and STAT3 have overlapping DNA binding sites [Zhang and Fuller, 1997]. Third, other polypeptides can induce STAT3-directed transcription, such as epidermal growth factor [Stahl et al., 1995]. Thus, it is reasonable to propose that while IL-6 signaling is functionally important, it is not absolutely necessary for normal development. Alternative pathways are capable of compensating for neutralized IL-6 signaling, as has been shown with liver regeneration [Sakamoto et al., 1999]. This testifies to the parallel nature and interconnectedness of signal transduction pathways [Weng et al., 1999].

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