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Expression and Regulation of Fas Antigen and Tumor Necrosis Factor Receptor Type I in Hen Granulosa Cells[1]

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ABSTRACT

It is now well established that vertebrate ovarian follicles undergo atresia via apoptosis, a process that is initiated within the granulosa cell layer of undifferentiated follicles. Although the exact signals, membrane-bound receptors, and associated intracellular signaling pathways leading to apoptosis within granulosa cells have yet to be established, it is evident that multiple and redundant pathways exist. Fas, together with its ligand, has been the most commonly studied death-inducer in the mammalian ovary; however, nothing is currently known regarding expression of either Fas or the related tumor necrosis factor receptor type 1 (TNFR1), in avian species. Based on characterization of a chicken fas partial cDNA, which includes the entire death domain, the deduced amino acid sequence shows 37% identity (53% positive) to human Fas. Northern blot analysis demonstrates low expression of the 2.0-kilobase fas transcript in most tissues, including the granulosa layer, and highest levels are found in the spleen, theca tissue, and the postovulatory follicle. Significantly, fas and tnfr1 mRNA levels are higher in atretic follicles than in nonatretic, prehierarchal (3- to 8-mm diameter) follicles. Moreover, both fas and tnfr1 mRNA levels are upregulated by twofold to eightfold in granulosa cells following plating in the presence of fetal bovine serum, with the most dramatic increase found in fas expression within prehierarchal follicle granulosa. Coculture with transforming growth factor (TGF) β attenuates this increase for both receptors, whereas cAMP attenuates only the up-regulation of fas. By comparison, treatment with TGFα enhances expression of tnfr1, but not fas, mRNA. Taken together, these data are the first to implicate fas as a mediator of granulosa cell apoptosis in a nonmammalian vertebrate, and to implicate the protein kinase A signaling pathway in down-regulating fas expression. In addition, data provided demonstrate the presence of multiple death domain-containing TNFR family members simultaneously expressed within hen granulosa cells, each of which may be regulated by separate signaling pathways.

apoptosis, follicle, granulosa cells, ovary, theca cells

INTRODUCTION

Only a small percentage of germ cells present within the vertebrate ovary survive to the ovulatory stage, whereas most degenerate via the process of atresia. Although many germ cells are lost prior to birth or hatch [1, 2], continued loss occurs throughout the reproductive life span of the female via follicle atresia. There is now ample evidence that the loss of developing ovarian follicles is initiated by the granulosa cell layer that surrounds the oocyte [3]. A distinct advantage of the avian model system is the ability to isolate pure populations of granulosa cells from atresia-susceptible prehierarchal follicles, as well as from atresia-resistant preovulatory follicles [4]. Moreover, granulosa cells from hen prehierarchal follicles have been reported to be susceptible to apoptosis when cultured for as little as 6 h in vitro, as evidenced by initiation of caspase activity and oligonucleosome formation, while preovulatory follicles remain resistant to apoptosis for more than 24 h under identical culture conditions [5]. Significantly, granulosa cell apoptosis can be attenuated by treatment with gonadotropins (acting via accumulation of cAMP) as well as a variety of growth factors, including transforming growth factor α (TGFα) [4, 6]. Growth factor activation of the PI3-kinase/Akt signaling pathway has recently been implicated in granulosa cell survival [7]. On the other hand, essentially nothing is known about endogenous signals and mechanisms leading to the initiation of hen follicle atresia, and more specifically, granulosa cell apoptosis.

Members of the tumor necrosis factor receptor (TNFR) family of death domain-containing receptors have been implicated in promoting cell death in variety of tissues. These membrane-anchored receptors are activated following ligand binding, and are coupled to caspase activation (caspase-8 in particular) by adaptor proteins such as Fas-associated death domain (FADD) or TNF receptor-associated death domain (TRADD) [8, 9]. Considerable evidence exists to implicate Fas-mediated signaling leading to apoptosis in mammalian ovarian granulosa cells [10–15]. Fas and Fas ligand have been localized to the granulosa layer within rat ovarian follicles that have been induced to undergo atresia in vivo by gonadotropin withdrawal [14]. Fas-mediated death in vitro is reported to occur following serum withdrawal and, depending on the species, may require cotreatment with interferon γ (IFNγ) and tumor necrosis factor α (TNFα), or cycloheximide [11–13].

The recent availability of chicken expressed sequence tag (EST) databases [16, 17] has enabled us to clone portions of the avian homologues to several death domain-containing TNF-family receptors, including Fas, TNF receptor type I (TNFR1), and death receptor 6 (DR6). Together with the previously identified p75NGFR [18] and CAR1 [19] receptors, there are now five death domain-containing receptors identified in the chicken, all of which are known to be expressed in ovarian granulosa cells [20]. With these newly identified genes, the opportunity exists to compare homology relative to their mammalian counterparts, to examine expression of fas and tnfr1 in atretic relative to viable follicles, and to evaluate regulation of the transcripts in response to cell survival factors.

MATERIALS AND METHODS

Animals and Reagents

Single-comb white Leghorn hens (H&H Poultry, Portland, IN), 25–35 wk of age and laying in regular sequences of at least five–six eggs, were

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used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layeena Mash, Purina Mills, St. Louis, MO) and water, and were exposed to a photoperiod of 15L:9D, darkness, with lights-on at midnight. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed approximately 16–18 h prior to a midsequence ovulation by cervical dislocation. All procedures described within were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee, and were performed in accordance with the *Guiding Principles for the Care and Use of Laboratory Animals.*

Recombinant human TGFα, TGFβ, and insulin-like growth factor I (IGF-I) were obtained from PeproTech (Rocky Hill, NJ). Ovine LH (lot 26) and recombinant human FSH (lot AP8468A) were provided by the National Hormone and Pituitary Program; 8-bromo-cAMP (8br-cAMP) was from Sigma Chemical Co. (St. Louis, MO). Treatment doses for culture experiments were based on previously published studies [4, 7].

Isolation of Chicken fas and Soluble fas Partial cDNA

The chicken fas was initially cloned using nested polymerase chain reaction (PCR) with primers based on a homologous sequence obtained from an activated T cell library EST (GenBank accession number AI981685 [16]). The double-stranded cDNA template, made from poly(A)$^+$ mRNA isolated from combined preovulatory and prehierarchal granulosa cells, was ligated to adaptors using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA). Initial amplification used a gene-specific primer, fas3′GSP1 (5′-GGG GAC TCT TCC ACC TGC TCC-3′), and the adaptor primer (AP1) with touchdown PCR conditions [21] using the Expand Long Template PCR system (Boehringer-Mannheim, Indianapolis, IN). An aliquot of the resulting cDNA was further amplified using a nested PCR reaction with the primers fasF1 (5′-ACA GTT TCA GTG GTC AGT GCT GC-3′) and fasB1 (5′-GCA ATA GAC ATA ATG CGT GCC C-3′), which yielded two different PCR products. Each was cloned into TA Cloning vector pCR2.1 and transfected into INVαF′ cells (Invitrogen, Carlsbad, CA). Products were subsequently sequenced using the dideoxychain-termination method and Sequenase version 2.0 (Amersham Pharmacia Biotech, Piscataway, NJ), and identified as the putative soluble and membrane-associated forms of Fas.

Rapid amplification of cDNA ends (RACE) reactions were performed in the 3′ direction to define the region of fas containing the putative death domain. The same template was employed initially using fas3′GSP1b (5′-CAG TTT CAG TGG TCA GTG CTG C-3′) and AP1 primers, followed by nested RACE reactions using fas3′GSP2 (5′-GCA CTC GGT TTG GAG GTT GTA AAG) and AP2 primers.

Evaluation of relative amounts of the soluble compared to transmembrane domain-containing transcripts in various tissues was accomplished using PCR with primers surrounding the transmembrane domain deletion. The primers were fasTM F1 (5′-CTT CTC GGT GTG AAC ATT GCG-3′) and fasTM B1 (5′-GCT GGT GGG TCA GGT CAA CAT C-3′). PCR (29 cycles at 94°C for 30 sec, 67°C for 30 sec, and 72°C for 1 min) was performed with cDNA templates made from 1 μg of total RNA collected from whole atretic and normal prehierarchal follicles, and from granulosa cells of prehierarchal and preovulatory follicles freshly collected or cultured with FBS (Gibco-BRL, Rockville, MD) for 20 h in the absence or presence of 8br-cAMP or TGFβ. The products were electrophoresed on a 1.5% agarose gel to differentiate the 300-base pair (bp) transmembrane domain-containing product from the 219-bp soluble form.

Isolation of a tnfr1 Partial cDNA

A partial cDNA encoding tnfr1, including the entire putative death domain, was isolated using homologous primers based on an EST sequence from a chicken fat cDNA library, *pBS*II (accession number AW35430). Total RNA from spleen tissue was used to make the cDNA template, which was amplified using the forward primer 5′-CAT CTC GGT GGA AAG AGT TTG TG-3′ with the reverse primer 5′-AGG AGG GTT AGA AAG ACA GGA AGG-3′ to yield a 353-bp product corresponding to the portion of tnfr1 that includes the death domain of human tnfr1 (accession number AAA36756). Amplification conditions were 94°C for 30 sec, a 56°C annealing temperature for 30 sec, and 72°C elongation for 1 min, for a total of 35 rounds, using Fisher *Taq* polymerase (Fisher Scientific, Chicago, IL). The PCR product was cloned into the TA cloning vector pCR2.1, and the nucleic acid sequence homology to mammalian tnfr1 was assessed, as described above.

Tissue Collection

Tissues collected for analysis of fas and tnfr1 mRNA expression included oviduct, brain, spleen, adrenal, and bone marrow. Ovarian samples consisted of the granulosa and theca tissue from the largest (F1), second largest (F2), and third largest (F3) preovulatory follicles, and prehierarchal (3–5 mm and 6–8 mm) follicles, together with ovarian stromal tissue and the most recent postovulatory follicle (POF). Morphologically normal and early atretic follicles (3–8 mm) were processed without separating granulosa and theca layers. All samples were collected and prepared as previously described [22].

Northern Blot Analysis of fas and tnfr1 mRNA

Levels of fas and tnfr1 mRNA were evaluated using Northern blots containing 10–15 μg of total RNA isolated from tissues or cells using Trizol Reagent (Gibco-BRL). Blots were probed with the initial transmembrane domain-containing clone for fas (371 bp), and death domain-containing clone for tnfr1 (353 bp). Templates were random-prime labeled using the Megaprime DNA Labeling System (Amersham) and [^{32}P]dCTP (3000 Ci/mmol; Amersham). Prehybridization was carried out for at least 30 min at 60°C, at which time probes were added and hybridization was allowed to proceed overnight. Stringent washes were conducted with two 5-min washes in 2× SSC (single-strength SSC = 150 mM sodium chloride and 15 mM sodium citrate pH 7.0) at room temperature, two 15-min washes in 2× SSC and 1% SDS at 60°C, and one 15-min wash with 0.1× SSC and 0.1% SDS at 60°C; followed by exposure to phosphorimaging plates for 1–2 wk. Images were captured using the Storm 840 PhosphorImager system equipped with the ImageQuant data reduction system (Molecular Dynamics, Sunnyvale, CA). Blots were initially hybridized with fas, followed by hybridization to the tnfr1 probe. When indicated, blots were subsequently reprobed with chicken 18S ribosomal RNA to standardize for loading of RNA.

Granulosa Cell Cultures

Granulosa cells from preovulatory (F1, F2, plus F3) follicles and prehierarchal (6–8 mm) follicles were collected, combined within their respective group, and dispersed in 0.3% collagenase (type 2; Worthington, Freehold, NJ) prior to treatment. An aliquot of each group was frozen immediately at −70°C (T0 controls), while the remaining cells were plated at 40°C in 6-well polystyrene culture plates (Falcon 3046; Fisher Scientific) at a density of approximately 10^6/well in 1 ml medium (M)199-HEPES supplemented with Hank salts (Gibco-BRL) plus 1 ml Dulbecco modified Eagle medium (DMEM) containing 5% FBS.

Experiments were performed to evaluate effects of gonadotropin or growth factor treatment on levels of fas and tnfr1 mRNA expression. First, prehierarchal granulosa cells were cultured for 6 h or 20 h in the absence or presence of 8br-cAMP (1 mM), FSH (100 ng/ml), LH (100 ng/ml), TGFβ (50 ng/ml), TGFα (50 ng/ml), or IGF-I (50 ng/ml). Media plus cells were collected from each well, and cells were pelleted by centrifugation at 200 × *g* for 5 min. Cells were frozen at −70°C until RNA was prepared for Northern blot analysis. A similar set of experiments was conducted with preovulatory follicle granulosa cells to evaluate potential differences in response relative to stage of differentiation.

Data Analysis

All experiments were replicated a minimum of three times, each using an individual chicken, unless otherwise stated. When appropriate, levels of fas and tnfr1 mRNA were standardized to 18S ribosomal RNA and expressed as the fold difference compared to freshly collected control (T0, arbitrarily set to 1) cells. Data were analyzed by one-way ANOVA without including data from the T0 group, and the Fisher Protected Least Significant Difference multiple range test. Further analysis of T0 versus T6 or T20 h samples (e.g., Figs. 6 and 7) was conducted by paired *t*-test using original data. Data from normal versus atretic follicles were compared using an unpaired *t*-test.

RESULTS

Alignment of TNFR Family Death Domains

A comparison of the deduced amino acid sequence for the death domains from the five chicken TNFR family members expressed within the ovary, Fas (Apo-1, CD95), TNFR1 (p55TNFR), DR6 (unpublished observations),

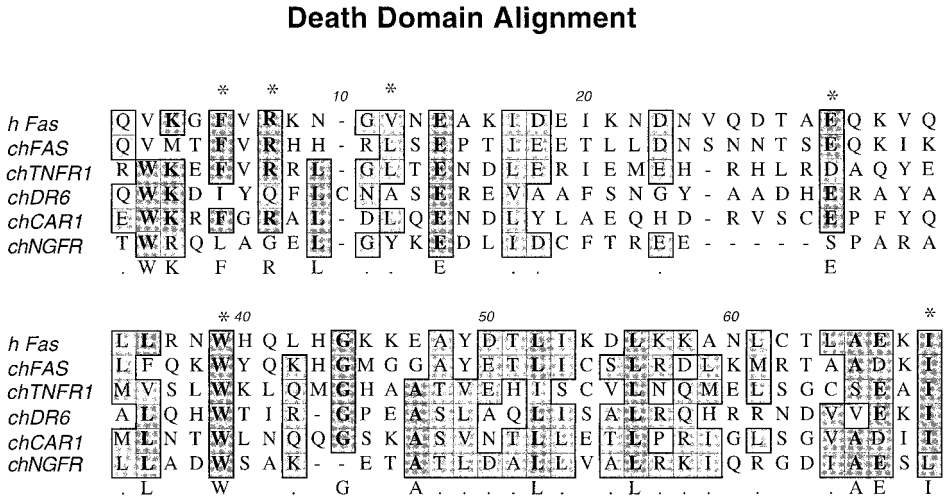

FIG. 1. Alignment of death domains for the five TNFR family members thus far identified in hen granulosa cells. Comparisons are based on the deduced amino acid sequences from chicken (ch) Fas (accession number AF296874), TNFR1 (accession number AW355430), DR6 (accession number AF349908), CAR1 (accession number AF161712), and p75NGFR (accession number P18519), compared with human (h) Fas (accession number AAA63174.1). Dark boxes indicate identical amino acids; lightly shaded boxes represent conserved substitutions. Asterisks indicate experimentally determined loss-of-function proteins following single amino acid substitution [23]. Consensus sequence is shown at the bottom.

CAR1 (TVB^{S3}) [19], and p75NGFR (p75NTR) [18], is shown in Figure 1. The death domain region is defined according to experimental data based on nonfunctional deletion mutants [23]. The degree of homology within the death domain varies between each individual receptor and its human counterpart, with amino acid identities of 43% for Fas, 47% for TNFR1, and up to 98% for DR6 [24]. The p75NGFR is the most divergent at functionally important amino acids, although general similarity to the death domain region is apparent.

Identification of an Alternatively Spliced Fas Soluble Form

During the process of amplifying the chicken fas sequence using primers designed against the EST sequence, a soluble form containing a transmembrane domain deletion was identified (Fig. 2). Amplification by PCR shows that relative levels of mRNA for the soluble compared to transmembrane domain-containing forms of fas are comparable under identical amplification conditions, whether from whole healthy or atretic follicles, or from granulosa cells derived from prehierarchal (atresia-susceptible) or preovulatory (atresia-resistant) follicles. Levels of mRNA increased following a 20-h culture in FBS, but there was no apparent differential regulation of the soluble versus transmembrane domain-containing forms of fas.

Expression of fas and tnfr1 mRNA

Northern blot analysis of chicken fas mRNA revealed a single, 2.0-kilobase (kb) transcript expressed largely in tissues of immune system or ovarian tissue origin at relatively low abundance. Tnfr1 mRNA demonstrated a similar pattern of expression among tissues, with an apparent doublet at approximately 3.0 kb (Fig. 3).

In the ovary, fas and tnfr1 mRNAs were expressed relatively evenly across stages of follicle differentiation (Fig. 4). Of significance, Northern blots of total RNA from whole, prehierarchal healthy or atretic follicles revealed significantly higher levels of both death domain-containing receptors in atretic follicles (fas, atretic 3.3 ± 0.4-fold higher compared with normal; tnfr1, atretic 2.0 ± 0.2-fold higher compared with normal; $P < 0.05$) (Fig. 5).

Cultures of Granulosa from Prehierarchal (6- to 8-mm Diameter) Follicles

Levels of fas transcript increased following cell culture in the presence of medium containing FBS by 6 h (2.5 ± 0.4-fold versus T0) and were further increased at 20 h (8.4 ± 1.4-fold versus T0; Fig. 6). The increase at 20 h was attenuated when the cells were plated in the presence of 8br-cAMP or TGFβ ($P < 0.05$). No significant effects compared to T20+FBS were observed when cells were cultured with FSH, LH, TGFα, or IGF-I. Levels of tnfr1 mRNA also increased following plating (2.0 ± 0.4-fold at T6, and 4.0 ± 0.4-fold at T20 versus T0 control), however, the fold increase at T20 was approximately half that seen for the fas mRNA. While TGFβ attenuated the increase in tnfr1 compared to T20+FBS, TGFα treatment further increased levels of the tnfr1 transcript (1.5 ± 0.2-fold versus T20 FBS; $P < 0.05$). Levels of tnfr1 mRNA did not change following treatment with 8br-cAMP, FSH, LH, or IGF-I (Fig. 6).

Cultures of Granulosa Cells from Preovulatory follicles

Fas mRNA levels were increased in preovulatory granulosa cells by 6 h (2.2 ± 0.3-fold versus T0) of culture and remained high when plated in the presence of FBS for up to 20 h (3.3 ± 0.4-fold versus T0; Fig. 7). This increase was attenuated by the presence of 8br-cAMP, FSH, LH, and TGFβ. By contrast, TGFα and IGF-I had no significant influence on fas mRNA levels. Expression of the tnfr1 transcript in preovulatory follicle granulosa cells also increased by a modest amount at 6 h (1.6 ± 0.2-fold versus T0) and by 2.8 ± 0.4-fold at T20 ($P < 0.05$), with no discernable treatment effects by 8br-cAMP, FSH, LH, TGFβ, or IGF-I. As in prehierarchal follicle granulosa cells, TGFα treatment significantly increased the levels of tnfr1 transcripts above the cultured control (2.4 ± 0.2-fold versus T20 control).

DISCUSSION

The results provided are the first to document expression of fas and tnfr1 in a nonmammalian ovary. Partial cDNAs encoding chicken homologues for the fas antigen and tnfr1 were identified, and on the basis of deduced amino acid sequences, they show 37% identity (53% similarity) to Fas and 42% identity (64% similarity) to TNFR1 compared with the respective human homologues. A total of five TNFR-family

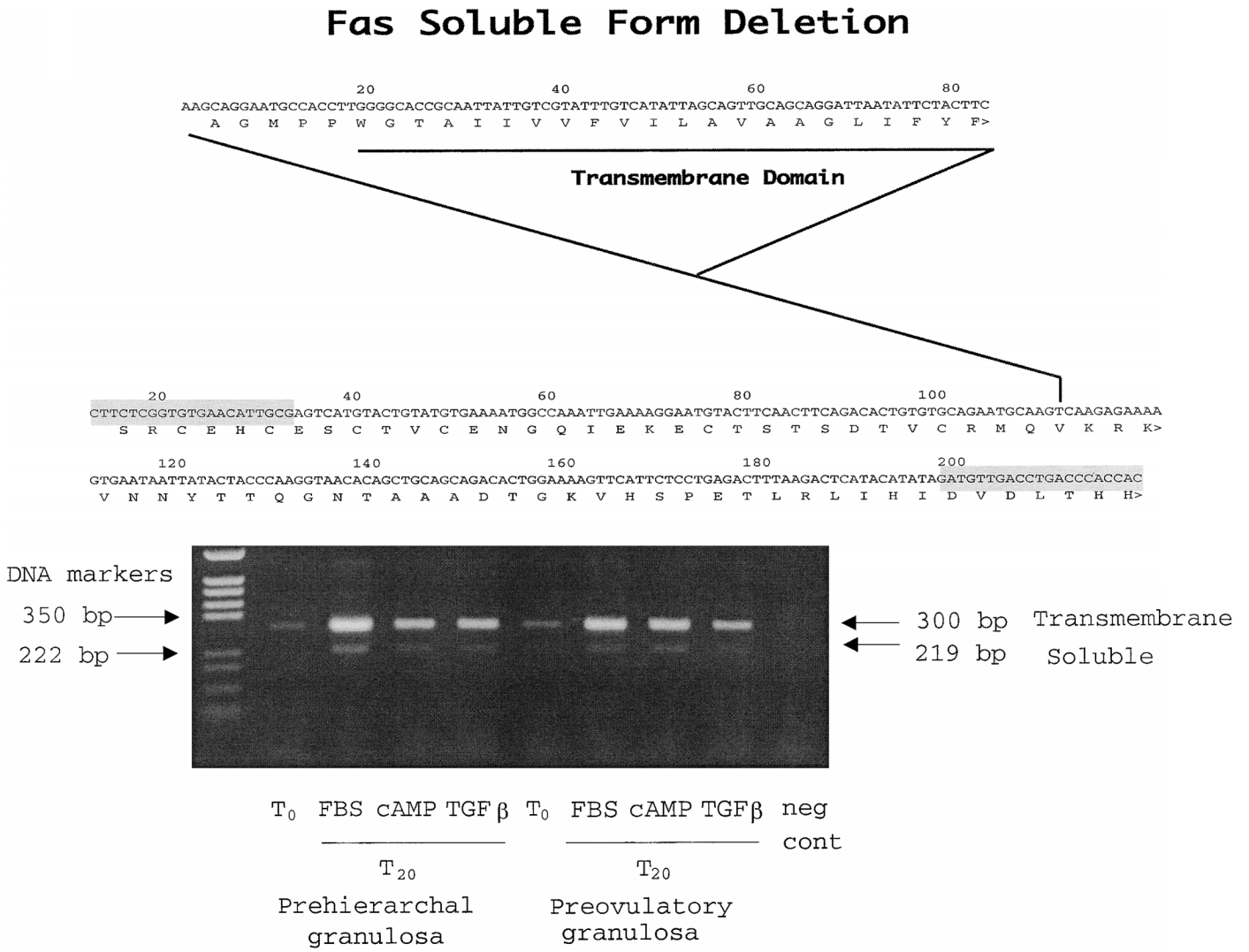

FIG. 2. The nucleic acid sequence from chicken fas was used to design primers (shaded) to amplify the region surrounding the transmembrane domain-containing region (underlined). The soluble form (lacking the transmembrane domain) corresponds to the transcriptional deletion found in the human fas mRNA. Amplification of prehierarchal and preovulatory follicle cDNA did not reveal obvious differences in the relative amounts of soluble versus transmembrane domain-containing transcripts from freshly collected tissue (T0) or after plating for 20 h (T20) with FBS, in the absence or presence of cAMP (1 mM) or TGFβ (50 ng/ml). The negative control (neg cont) contained no cDNA template. This experiment was repeated once with similar results.

death domain-containing receptors have now been identified in the chicken, and each is expressed in granulosa cells. These receptors vary in their degree of homology to their human counterparts (a CAR1 homologue has not yet been identified in mammalian species), with amino acid identity of the functional death domain-containing region ranging from 43% for Fas to 98% for DR6. The low level of identity for the deduced chicken sequence for Fas was not unexpected given the considerable divergence between mammalian Fas receptors (e.g., human [accession number AAA63174] and rat [accession number BAA05108] Fas death domains are only 52% identical). It is interesting that although the chicken p75NGFR death domain region is similar to that of other TNFR-family members, the conservation of sequence homology does not include five of six residues deemed important for its death-inducing function as determined by in vitro mutagenesis studies (Fig. 1; [23]). Perhaps not unexpectedly, the p75NGFR has been shown to mediate neuronal death through an as-yet-unknown signaling pathway that apparently does not involve the death domain [25]. On the other hand, activation of the p75NGFR using avian nerve growth factor (NGF), neurotrophin-3, or brain-derived neurotropic factor fails to initiate death in cultured hen granulosa cells (unpublished data).

Transcripts for both fas and tnfr-1 are expressed in both granulosa and theca cells of hen ovarian follicles, as well as in tissues of immune system origin [26, 27]. As previously reported for the fas antigen in rat ovaries [14, 28], both fas and tnfr1 are expressed at elevated levels in atretic follicles, relative to healthy hen follicles. Such increases in expression provide evidence for a causative effect of these receptors with apoptotic cell death, and by implication, follicle atresia, in vivo.

In addition to a membrane anchored form of fas, a soluble form of the chicken fas receptor was identified. The sequence deleted from the soluble form consists of 81 nucleic acids that are spliced from the identical location as the soluble transmembrane deletion (TM-del) form of the human fas gene [29, 30]. Given the small size of the deletion it is not surprising that the soluble form is not distinguishable from the membrane-anchored form on Northern blots in which a single, 2.0-kb signal appears. The hu-

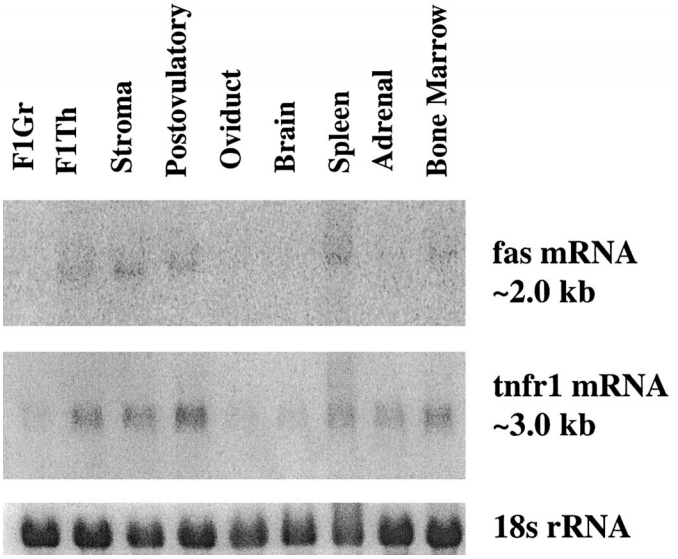

FIG. 3. Northern blot analysis of fas (top panel) and tnfr1 (bottom panel) mRNA expression in various hen tissues, with 18S ribosomal (r) RNA provided for evaluation of equal loading. F1Gr and F1Th, granulosa and theca, respectively, from the largest preovulatory follicle; stroma, ovarian stromal tissue; brain, cerebrum. Each blot was replicated once with similar results.

man TM-del was characterized as a transcriptional splicing variant, and has since been shown to produce a secreted form of Fas that can be measured in serum. This soluble form is capable of inhibiting Fas-mediated apoptosis in vitro [29], and levels of serum soluble Fas correlate well with advanced stages of gynecological cancers [31]. Amplification by PCR demonstrated the presence of both forms of Fas mRNA following culture (Fig. 2).

TNF family receptors have been proposed to play a role in the initiation of ovarian follicular atresia in mammalian species. More specifically, Fas antigen and TNFR1 have been implicated in granulosa cell apoptosis. One line of evidence provided by transgenic mouse models includes the mouse mutant lpr (lymphoproliferation) containing a mutation in the gene encoding the Fas antigen. The young lpr

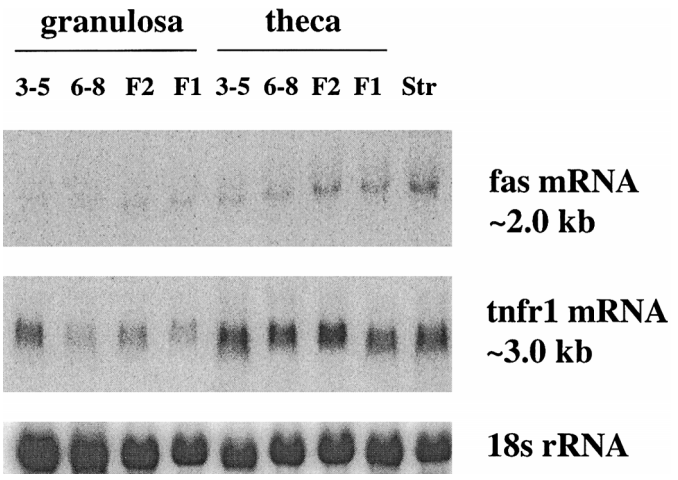

FIG. 4. Expression of fas (top) and tnfr1 (bottom) mRNA in granulosa and theca tissue during follicle development, with 18S rRNA provided as a control for loading. F2 and F1 represent the second largest and largest preovulatory follicles, respectively; 3–5 and 6–8 are follicle size in mm; Str, ovarian stromal tissue. These blots were replicated once with similar results.

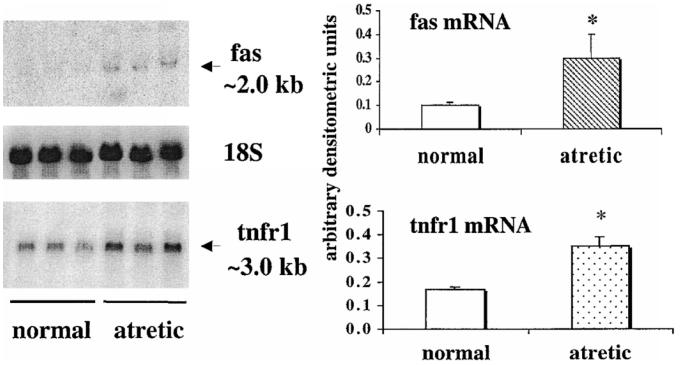

FIG. 5. Levels of fas (top) and tnfr1 (bottom) mRNA isolated from whole prehierarchal healthy (normal) versus atretic follicles. Summarized data represent the mean ± SEM, standardized to levels of 18S rRNA. $*P < 0.05$ by *t*-test.

mutant mice had morphologically normal ovaries, however, adult mice had a significantly larger number of growing follicles than wild-type mice [32]. These adult mice were shown to have larger ovaries due to the increased numbers of follicles present [33], which suggested an important role for Fas in regulation of follicle survival and development of the ovary. By comparison, TNFR1 knockout mice exhibited early senescence and poor fertility, in part due to TNFR1-mediated inhibition of steroidogenesis [34]. The

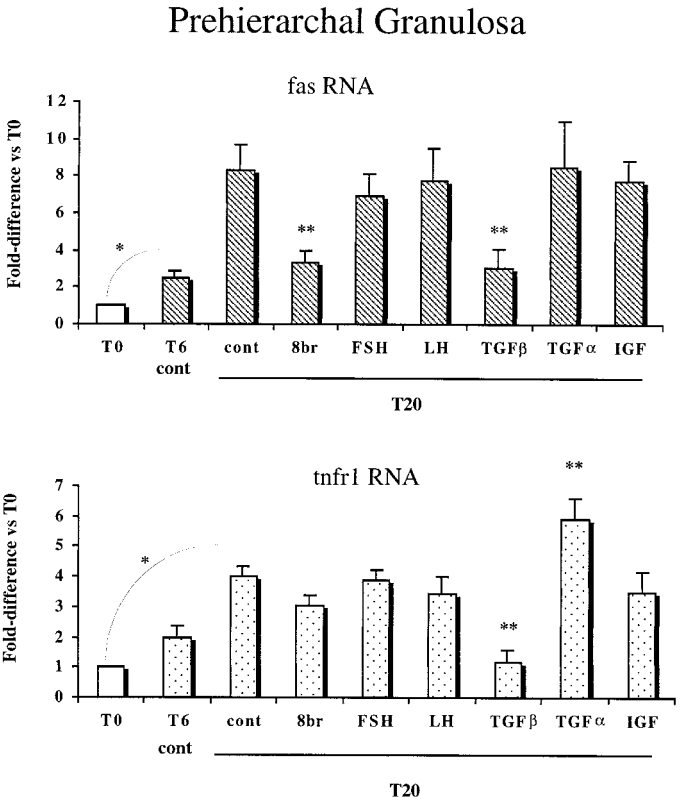

FIG. 6. Evidence for regulation of fas (top) and tnfr1 (bottom) mRNA expression in cultured prehierarchal follicle granulosa cells. Granulosa cells were cultured for 6 or 20 h in the absence (con) or presence of 8br-cAMP (8br; 1 mM), FSH (100 ng/ml), LH (100 ng/ml), TGFβ (50 ng/ml), TGFα (50 ng/ml), or IGF-1 (50 ng/ml). Values for fas and tnfr1 are adjusted for loading with 18S rRNA and expressed as the fold difference versus freshly collected (T0) cells. Data represent the mean ± SEM from three to five experiments. $**P < 0.05$ relative to T20 control by ANOVA; $*P < 0.05$ versus T0 by paired *t*-test.

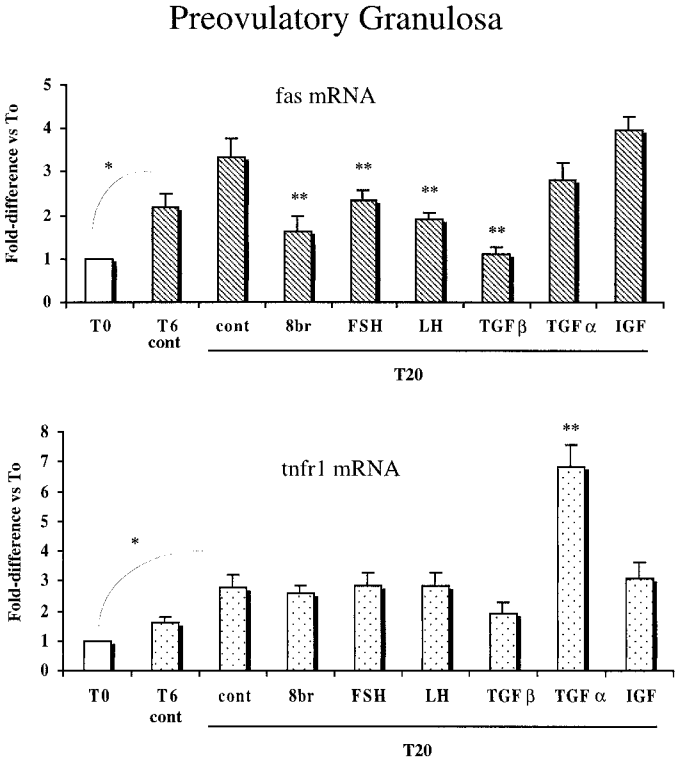

FIG. 7. Evidence for regulation of fas (top) and tnfr1 (bottom) mRNA expression in cultured preovulatory follicle granulosa cells. Granulosa cells were culture for 6 or 20 h in the absence (con) or presence of 8br-cAMP (8br; 1 mM), FSH (100 ng/ml), LH (100 ng/ml), TGFβ (50 ng/ml), TGFα (50 ng/ml), or IGF-1 (50 ng/ml). Values for fas and tnfr1 are adjusted for loading with 18S rRNA and expressed as the fold difference versus freshly collected (T0) cells. Data represent the mean ± SEM from three to five experiments. $^{**}P < 0.05$ relative to T20 control by ANOVA; $^{*}P < 0.05$ versus T0 by paired *t*-test.

lack of TNFR1 signaling in these mice resulted in early onset of follicle development, perhaps due to heightened sensitivity to gonadotropin in young mice. This response was not seen in adult mice and suggested a stage-specific role for TNFR1 signaling in the ovary. Further studies using rat ovarian follicles treated with FSH showed that TNFα induced apoptosis through the ceramide signaling pathway [35]. Significantly, a stage-specific response was also observed in the hen ovary, in which recombinant mouse TNFα potentiated oligonucleosome formation in prehierarchal, but not preovulatory, follicle granulosa cells [6].

Results of culture experiments demonstrated increased fas and tnfr1 mRNA expression in granulosa cells following plating, and these increases were more dramatic for the levels of fas mRNA in apoptosis-sensitive prehierarchal granulosa cells. The implication of this response to plating is that levels of fas and tnfr1 expression are under control of inhibitory signaling pathways in vivo, and that this inhibition is removed in culture. The lack of cell death in coordination with the increase in fas transcript may indicate that apoptosis is dependent upon other factors, such as the expression of fas ligand or the presence of apoptosis inhibitors. Treatment of granulosa cells with the cell-permeable cAMP analogue, 8br-cAMP, has previously been shown to provide the strongest survival effects on these cells [4, 6], and in the present experiments, attenuated the increased expression of fas mRNA. Moreover, preovulatory granulosa cells, which are known to express LH receptor and to a lesser extent FSH receptor [36, 37], also demonstrated an attenuation of fas up-regulation upon gonadotropin treatment with LH and FSH. On the other hand, although prehierarchal follicle granulosa cells express predominantly FSH receptor and respond to FSH treatment with increased cAMP accumulation [22], fas expression was not affected by FSH treatment. It is possible that the endogenous levels of cAMP induced by FSH signaling may not have been sufficient to attenuate the eightfold induction of fas transcript for the prehierarchal follicle granulosa. Alternatively, attenuation of fas expression could occur through the actions of vasoactive intestinal peptide (VIP), which is also known to represent a granulosa cell survival factor signal via protein kinase A signaling [38, 39].

Although TGFα and IGF-I have been shown to promote cell survival in hen granulosa cells [4, 7], neither growth factor influenced fas mRNA levels at either stage of follicle development. The cytokine, TGFβ, has previously been shown to inhibit basal levels of Fas expression in murine microglial cells, and to inhibit Fas-mediated death of these cells when cocultured with IFNγ or TNFα [40]. It is interesting that treatment with TGFβ attenuated the culture-induced increase in fas mRNA levels in both hen prehierarchal and preovulatory granulosa. At this time the significance of this observation is not clear because the role of TGFβ signaling in hen granulosa cell survival has yet to be studied.

Tnfr1 mRNA levels also increased following culture, and were attenuated by TGFβ treatment in granulosa cells from prehierarchal follicles. However, in contrast to fas levels, tnfr1 transcripts were not attenuated by gonadotropin or 8br-cAMP treatment at either stage of follicle development. Similarly, the absence of change in tnfr1 mRNA levels in response to cAMP was observed in human hematopoietic cells, which demonstrated posttranscriptional regulation of TNFR1 [41]. Alternatively, the ligand TNFα may be the site of regulation by gonadotropins rather than its receptor. By comparison, both prehierarchal and preovulatory follicle granulosa cells responded to TGFα treatment with increased tnfr1 mRNA expression. Without knowing more about the function of tnfr1 in this cell type, we cannot explain the significance of the TGFα treatments at this time.

In summary, five different death domain-containing receptors are now known to be expressed within granulosa cells of hen ovarian follicles. In the present study, two of these, fas and tnfr1, are shown to be expressed at higher levels in atretic relative to healthy follicles in vivo. Both receptors demonstrate increased expression following culture of prehierarchal and preovulatory follicle granulosa cells, and each can be regulated by gonadotropin and growth factor treatments. Although these data suggest a role for death domain-containing receptors in mediating hen follicle atresia, further research will be required to understand the conditions under which their activation results in programmed cell death and the signaling pathways through which this effect is mediated.

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