Avian TVB (DR5-like) Death Receptor Expression in Hen Ovarian Follicles

Jamie T. Bridgham and Alan L. Johnson
Department of Biological Sciences and Walther Cancer Research Center, University of Notre Dame,  
P.O. Box 369, Notre Dame, Indiana 46556

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TVB is an avian death domain-containing receptor belonging to the TNF receptor family and is proposed to be the ortholog to mammalian DR5. Although TVB receptor activation has been demonstrated to mediate apoptosis in chick embryo fibroblasts, there is essentially no information regarding TVB expression or regulation in the mature hen ovary, and in particular within the follicle granulosa layer where apoptosis is known to promote atresia. Significantly, the TVB receptor represents the fourth death domain-containing receptor (also including Fas, TNF-R1, and DR6) found to be expressed within hen granulosa cells. Levels of TVB expression are higher in prehierarchal follicles actively undergoing atresia compared to healthy follicles. However, increased TVB expression does not precede follicle death induced in vitro. Furthermore, TVB expression within granulosa cells is highest during the final stages of follicle development when follicles are not normally susceptible to undergoing atresia. These results provide evidence that TVB receptor signaling in the ovary may function in a capacity other than solely to mediate granulosa cell death and follicle atresia. © 2002 Elsevier Science (USA)

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Programmed cell death (apoptosis) is the physiological process by which organisms eliminate excessive or nonviable cells and tissues (1). Pathways mediating apoptosis may be initiated either by intrinsic mechanisms (via mitochondrial perturbations) or by extrinsic factors (via receptor-mediated events). The tumor necrosis factor receptor (TNF-R) family currently includes at least six members that contain conserved death domains that are critical to promoting apoptosis in response to extrinsic (both physiological and viral) factors. Included among these receptors is the avian TVB (CAR1) receptor which was originally identified as a result of its ability to bind envelope coat proteins from cytopathic avian leukemia-sarcoma viruses (ALV) (2). Treatment of cultured chick embryo fibroblasts with a recombinant ALV envelope fusion protein (SUB-rIgG) induces apoptosis (2), and such induced cell death is dependent upon the presence of a functional TVB death domain (3). TVB is proposed to represent the avian ortholog to one of two human TRAIL receptors, DR4 (TRAIL-R1) or more likely DR5 (TRAIL-R2), by virtue of a 30 and 37% amino acid sequence identity, respectively (4). Moreover, within the functional death domain, residues determined to be critical for death-inducing function of DR5, by loss-of-function or partial loss-of-function mutations (5) are either identical or conserved substitutions when compared to the sequence of the avian TVB protein.

It is well established that the death of follicles (follicle atresia) occurring during development in both the mammalian and avian ovary is mediated by the initiation of apoptosis within the granulosa cell layer (6, 7). There is now considerable information derived from in vitro studies regarding the ability of TNF-R1 and the related family member, Fas, acting via TNF-α and Fas ligand, respectively, to promote granulosa cell death in mammalian species (8–10). Similarly, we have recently described the expression of Fas, TNF-R1 and Death Receptor-6 (DR6) in hen granulosa cells (11, 12). Nevertheless, the physiological conditions under which such receptors actually trigger granulosa cell death, as well as whether they exist solely to terminate cell viability, remain to be determined. Moreover, the functional significance for multiple death domain-containing receptors expressed by ovarian granulosa cells during follicle development remains largely unstudied in either avian or mammalian species. In this communication we report patterns of tvb mRNA and TVB protein expression within hen ovarian follicles during de-
velopment and describe TVB regulation by growth factors and gonadotropins in cultured granulosa cells. Results presented demonstrate that increased TVB receptor expression is not necessarily associated with the initiation of follicle death or with granulosa cell susceptibility to apoptosis. These findings suggest that the avian TVB receptor in the ovary may mediate functions other than those related solely to the initiation of apoptosis.

MATERIALS AND METHODS

Animals and reagents. Single-comb white Leghorn hens (Creighton Brothers, Warsaw, IN), 25–35 weeks of age and laying regular eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layena Mash, Purina Mills, St. Louis, MO) and water, and were exposed to a photoperiod of 15 h light, 9 h 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 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 transforming growth factor-α (TGF-α) was obtained from PeproTech (Rocky Hill, NJ). Actinomycin D was from Gibco-BRL (Rockville, MD) while β-galactosidase (β-gal), and Freund’s Complete and Incomplete Adjuvant were obtained from Sigma Chemical Co. (St. Louis, MO). Ovine lutenizing hormone (LH; 3000 CI/mml; Amersham Pharmacia Biotech, Piscataway, NJ) using terminal transferase (Roche Molecular Biochemicals, Indianapolis, IN). Gels were analyzed by autoradiography and subsequent quantitation of β-subunit or full length β-adrenergic receptor protein by phosphorimaging (Storm 840 PhosphorImager system equipped with the ImageQuant data reduction system (Molecular Dynamics, Sunnyvale, CA). All blots were subsequently reprobed with an 18S ribosomal RNA probe to enable standardization for RNA loading.

Northern blot analysis for tvb. Levels of tvb mRNA were evaluated from 15 μg of total RNA isolated from tissues or cells using Trizol Reagent (Gibco-BRL). A cDNA template was prepared by amplifying a 545 bp fragment (nucleic acids 503–957) based upon the published sequence for chicken tvb (2; Accession No. AF161712). This polymerase chain reaction (PCR) product was ligated into the pCR2.1 cloning vector (Invitrogen, Carlsbad, CA), and verified by nucleic acid sequencing (Fas paper, Sequenase ver. 2.0, USB, Cleveland, OH) prior to use. The template was random-prime labeled using the Megaprime DNA Labeling System (Amersham) and T7 (2000 CI/mml; Amersham). Stringent hybridization and washing was performed at 60°C as previously described (11). Images were captured using the Storm 840 PhosphorImager system equipped with the ImageQuant data reduction system (Molecular Dynamics, Sunnyvale, CA). All blots were subsequently reprobed with an 18S ribosomal RNA probe to enable standardization for RNA loading.

Western blot analysis using a SUB-ribG fusion protein and polyclonal antibody. A rabbit polyclonal antisemur was generated against chicken amino acids 56–164 located within the extracellular domain of the published tvb sequence (2). To produce the antigen, a cDNA PCR product coding for nucleic acids 167–492 was amplified, ligated into the pCAL-n vector, and transformed into BL21 (DE3) plYS Competent cells (Stratagenex, La Jolla, CA). Western blot analysis was performed using a 10% acrylamide gel, then mixed 1:1 with Complete Freund’s adjuvant (first injection) or Incomplete Freund’s adjuvant (booster injections) for immunization (100 μg fusion protein per injection). Antibody specificity was verified both by comparison with preimmune serum and by using an affinity purified serum against the antigen.

Tissue collection. Tissues collected from the adult chicken included ovarian strona, oviduct, bone marrow, kidney, brain, spleen and heart. Ovarian follicle samples consisted of granulosa and theca tissue from the largest (F1), second largest (F2), and third largest (F3) preovulatory follicle, the follicle most recently recruited to the tissue from the largest (F1), second largest (F2), and third largest (F3) follicle. The follicle most recently recruited to the preovulatory hierarchy (9–12 mm in size), and prehierarchal (3–5 and 6–8 mm undifferentiated) follicles. Morphologically normal and atretic prehierarchal follicles (3–8 mm) were collected without separating the granulosa and theca layers, as previously described (13).

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 cells from each group was frozen immediately at −70°C (T0 controls), while the remaining cells were plated at 37°C in 6-well polystyrene culture plates (Falcon 3046; Fisher Scientific) at a density of approximately 10⁶/well in 1 ml medium (M199–Hepes supplemented with Hanks’ salts (Gibco-BRL) plus 1 ml Dulbecco’s modified Eagle medium (DMEM) containing 5% fetal bovine serum (FBS; Gibco-BRL).

In initial culture experiments, granulosa cells from prehierarchal or preovulatory follicles were plated in the absence or presence of actinomycin D (1 μg/ml) to monitor changes in tvb mRNA expression at increasing lengths of time following plating. Subsequent culture experiments were conducted to evaluate the effects of gonadotropin or growth factor treatment on levels of tvb mRNA or TVB protein expression. Oligonucleosome formation was monitored in parallel samples to assess the corresponding extent of apoptosis. Granulosa cells from prehierarchal or preovulatory follicles were cultured for 20 h at 37°C in the absence or presence of 8br-cAMP (1 mM), LH (100 ng/ml), FSH (100 ng/ml) or TGF-α (50 ng/ml). Treatment doses were based on previously published studies (11, 14). Medium plus cells were collected from each well, and cells were pelleted by centrifugation at 200g for 5 min. Cell pellets were frozen at −70°C until further processed for DNA, RNA or protein.

Preparation of genomic DNA for evaluation of oligonucleosome formation. Genomic DNA was isolated from treated tissues and controls as previously described (15). DNA (1 μg per lane) was separated on 1.8% agarose gels following 3′-end-labeling with [α-32P]-dideoxy-ATP (3000 Ci/mmm; Amersham Pharmacia Biotech, Piscataway, NJ) using terminal transferase (Roche Molecular Biochemicals, Indianapolis, IN). Genomic DNA was gel-purified using a 10% acrylamide gel, then mixed 1:1 with Complete Freund’s adjuvant (first injection) or Incomplete Freund’s adjuvant (booster injections) for immunization (100 μg fusion protein per injection). Antibody specificity was verified both by comparison with preimmune serum and by using an affinity purified serum against the antigen.

Tissue proteins were isolated and subsequently separated on a 10% acrylamide gel as previously described (12). Following transfer to a nitrocellulose membrane, the blots were blocked for 1 h in Western blocking solution (TBST: 10 mM Tris (pH 8.0), 150 mM sodium chloride, 0.1% Tween 20 containing 5% nonfat dry milk). Membranes were then incubated with either the TVB polyclonal antibody (described above) at 1:5000 dilution in Western blocking solution or with SUB-ribG fusion protein (1:4 dilution; provided by J. Young, University of Wisconsin). This fusion protein represents an ALV subtype B viral envelope protein fused to the Fc region of a rabbit immunoglobulin, and is known to bind with specificity to the TVB membrane receptor. In both instances, blots were incubated at 4°C overnight, then washed 3 times for 5 min in Western blocking solution followed by a 1–2 h incubation at RT in goat anti-rabbit serum: horseradish peroxidase conjugated secondary (Pierce Chem. Co., Rockford, IL) diluted 1:5000 in Western blocking solution. Nitrocellulose membranes were subsequently washed in TBST (3 times for 10 min each) and finally incubated with ECL Western blotting detection reagent (Amersham) for 1 min, and exposed to autoradiograph film for 20 s to 5 min. The extent of antibody binding was quantitated by densitometry (UltraScan XL laser densitometer, Pharmacia LKB, Piscataway, NJ), and routinely standardized to α-tubulin (12).
RESULTS AND DISCUSSION

The previously published description of relatively low and somewhat selective expression of tvb mRNA among largely embryonic tissues (2) prompted us to examine the distribution of tvb over a broader range of tissues from the adult hen. Little or no tvb expression is found in kidney, brain, and heart, but low levels of constitutive expression are detected in the bone marrow, spleen (data not shown), as well as in granulosa and theca tissues from the ovary. The observation that tvb mRNA is expressed within the hen ovary initially led to the hypothesis that an upregulation of TVB receptor expression could be directly associated with ovarian follicle atresia, and in particular, susceptibility to apoptosis in granulosa cells.

Previous studies have determined that preovulatory follicles are not normally subject to becoming atretic, in vivo, and that granulosa cells from preovulatory follicles are resistant to undergoing apoptosis, in vitro (15). Within the follicle granulosa layer, tvb mRNA levels are highest in 6- to 8- and 9- to 12-mm follicles, with comparatively lower levels in preovulatory (F3, F2, and F1) follicles (Fig. 1A, left panel). By comparison, tvb mRNA and TVB protein expression within the theca cell layer does not change during follicle development (Fig. 1B).

Surprisingly however, such stage-related changes in tvb mRNA within the granulosa layer do not reflect the pattern of TVB protein expression. Protein blot analysis found 3- to 10-fold more TVB protein in granulosa from F3 to F1 preovulatory follicles compared to 3-5 and 6-8 mm prehierarchical follicles (P < 0.05), whether detected using the TVB polyclonal antibody or the SUB-rIgG fusion protein (Fig. 1A, right panel). The discrepancy between mRNA and protein levels in granulosa during follicle development indicates that either factors within preovulatory follicles enhance protein stability resulting in an accumulation of higher levels of TVB, or that posttranscriptional regulation plays a role in TVB receptor expression. In this regard, the up-regulation of protein expression in the absence of increased mRNA for the related death receptors, DR4 and DR5, has been demonstrated following paclitaxel treatment of human prostate cancer cells (18); however, the mechanism of post-transcriptional receptor induction has not been elucidated. Moreover, when protein blots are incubated with SUB-rIgG fusion protein, multiple protein bands are detected compared to the single signal obtained using the TVB antiserum.

This difference is likely attributed to the ability of the SUB-rIgG fusion to recognize glycosylated forms of the protein, as previously noted (2). Significantly, the glycosylation status was determined not to be critical to death inducing ability of TVB (19).

In light of this pattern of TVB protein expression in granulosa cells from preovulatory follicles, it is reasonable to suggest that the upregulation of the TVB receptor is not necessarily related to cell death. For instance, mammalian DR5 signaling via nuclear factor-κB (NF-κB) and/or J un kinase activation is associated with cell survival rather than death (20, 21). Additionally, in a variety of cell types both TNF-RI and DR6 activate cell signaling via NF-κB which can result in the suppression of apoptosis (22, 23). Specifically, TNFα treatment of rat granulosa cells increases levels of the Inhibitor of Apoptosis Protein, XIAP, via activation of NF-κB (24). Clearly, the elucidation of TVB cell signaling pathways following receptor activation will be required in order to clarify its function in fully differentiated, apoptosis-resistant granulosa cells.

Conversely, granulosa cells from undifferentiated, prehierarchical follicles are highly susceptible to undergoing apoptosis, in vitro, and such follicles commonly undergo atresia, in vivo (15). Whole prehierarchical follicles undergoing atresia, in vivo, express higher levels of tvb mRNA (4.3-fold; P < 0.05) and TVB protein (2.8-fold; P < 0.05) compared to morphologically healthy follicles of comparable size (Fig. 2A). These findings provide a correlative relationship between elevated TVB expression and the initiation of follicle atresia, in vivo. In an attempt to replicate this relationship, in vitro, morphologically normal whole prehierarchical follicles were incubated in medium containing 2.5% FBS for 6 h, after which time the granulosa layer was isolated and processed for DNA or protein. Significantly, despite evidence for induced apoptosis within granulosa layers incubated for 6 h (Fig. 2B) there was no increase in TVB protein (Fig. 2C) preceding the onset of oligonucleosome formation. These findings suggest that increased TVB expression in atretic follicles, in vivo, may occur as a result of, and is not always required for, apoptotic cell death within prehierarchical follicles.

In an attempt to examine factors involved in regulating tvb mRNA expression specifically within the granulosa layer, cells from prehierarchical (6-8 mm) or preovulatory follicles were dispersed with collagenase and plated in the absence or presence of actinomycin D for up to 20 h. In contrast to granulosa from whole follicle incubations, tvb mRNA levels in dispersed prehierarchical follicle granulosa cells were rapidly increased by 9.3 ± 2.7-fold following 3 h of culture, and remained elevated up to 20 h following culture (7.4 ± 1.5-fold) (Fig. 3A). Levels of mRNA were also increased following culture of dispersed preovulatory follicle granulosa, but to a lesser extent (4.6 ± 0.7-fold following 3 h of culture; 4.2 ± 1.5-fold following 20 h culture) (Fig. 3B).
This increase in tvb represents newly synthesized RNA as coculture with actinomycin D inhibits this response. Moreover, this increase in tvb mRNA is accompanied by increased TVB protein expression in both prehierarchal follicle granulosa (a 15.4- ± 2.7-fold increase after 6 h; Fig. 4A) and preovulatory follicle granulosa (a 3.4- ± 1.1-fold increase after 20 h; Fig. 5A).

The increase in tvb mRNA expression observed following cell dissociation and culture is similar to that recently reported for fas and tnfr1 mRNA (11), and may occur in response to the loss of matrix protein signaling following detachment from the basement membrane. Although generally the role of death receptors in cell death via anoikis (a form of apoptosis trig-
gered by the loss of cell-matrix signaling) has yet to be firmly established (25), cell detachment has been observed to increase Fas mRNA expression in human umbilical vein endothelial cells (26), while a dominant negative form of the Fas adaptor protein, FADD, efficiently blocks anoikis in Madin–Darby canine kidney (MDCK) cells (27, 28). Nevertheless, in hen granulosa
cells this culture-induced increase in TVB expression is neither necessary for the initiation of apoptosis, as actinomycin D treatment fails to block culture-induced oligonucleosome formation in prehierarchical follicle granulosa cells (Fig. 3B, top), nor is it sufficient by itself to promote cell death as increased TVB expression is not associated with any significant oligonucleosome formation in preovulatory follicle granulosa after 20 h of culture (Fig. 3B, bottom). These findings are perhaps not unexpected given that a number of intracellular factors have been more directly linked to the susceptibility/resistance of hen granulosa cells to apoptosis. For instance, apoptosis-resistant granulosa cells from preovulatory follicles are known to express comparatively higher levels of the Inhibitor of Apoptosis Protein, cIAP1 (ITA; 14) plus the death-suppressing Bcl-2-related protein, Bcl-XLong (15) compared to apoptosis-susceptible cells from prehierarchical follicles.

Finally, cell survival factors that attenuate oligonucleosome formation in cultured prehierarchical follicles (8-bromo-cAMP, FSH and TGFα; Fig. 4B) attenuate the increase in TVB receptor expression following 20 h of culture (Fig. 4C). Similar results were obtained with granulosa cells from preovulatory follicles cultured in the absence or presence of cAMP and LH, although the extent of TVB modulation by survival factors is comparatively less in preovulatory follicle granulosa (Fig. 4C versus Fig. 5B). Once again, however, while it is of interest to demonstrate the ability of endocrine and paracrine factors to regulate TVB expression, the functional relationship between cell survival signaling and the modulation of TVB expression remains to be established.

In summary, these results document the expression of the avian TVB receptor within hen ovarian granulosa cells, and this represents the fourth death domain-containing receptor (including Fas, TNF-RI and DR6; 11, 12) thus far described in this ovarian epithelial cell type. Follicle atresia in vertebrates has been proposed to result: (i) from an active induction by endogenous cytokines or exogenous pharmacologic agents (e.g., chemotherapeutics); (ii) from the withdrawal of cell survival factors (e.g., gonadotropins, growth factors, or their respective receptors); or (iii) in response to inherent defects within the follicle that preclude the development of a viable germ cell (29). Although the physiologic relationship of death receptor expression to any of the above causes of ovarian follicle death, in vivo, has yet to be firmly established, it is reasonable to predict that such receptors collectively provide the capability of directly responding to a diverse repertoire of death-inducing extrinsic factors. Given that the avian TVB receptor is predicted to represent the ortholog to mammalian DR5 (TRAIL-R2), it will be of interest to determine whether the chicken TRAIL ligand binds to and activates TVB receptor signaling. Moreover, it is also possible that death receptor upregulation and signaling can occur following initiation of an intrinsic pathway, as a mechanism to amplify cellular apoptotic pathways and insure the complete removal of a tissue.

Of equal importance and based upon patterns of expression described herein, the TVB receptor is predicted to have multiple and diverse signaling roles within hen ovarian follicles during development. This is perhaps not unexpected given the multiple, complex signaling pathways already documented for the Fas, TNF-RI and DR6 receptors in extraovarian tissues (22, 23). Therefore, further studies will be required to fully understand the extent and conditions under which TVB is directly or indirectly involved in regulating ovarian follicle atresia, as well as what additional functions TVB assumes in ovarian follicles during follicle development and differentiation.

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