Granzymes D, E, F, and G Are Regulated Through Pregnancy and by IL-2 and IL-15 in Granulated Metrial Gland Cells

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Granulated metrial gland (GMG) cells are NK cells that proliferate and differentiate within the murine uterus during pregnancy. They have been predicted to play important roles in nurturing the embryo, normal placentation, and uterine tissue remodeling. GMG cell differentiation is manifested by the accumulation of the cytolytic mediators, perforin, granzyme A, and granzyme B, within cytoplasmic granules. The signaling mechanisms required for GMG cell differentiation are largely unknown, although recent in vitro assays have implicated IL-15 in these events. In this report, we demonstrate that granzymes D, E, F, and G (granzymes D–G) are also expressed in GMG cells but at a later stage in pregnancy when compared with granzyme A expression. Whereas granzyme A is expressed in early to mid-gestation, the expression of granzymes D–G peak in mid- to late gestation. In addition, we show that the expression patterns of IL-2Rβ and the IL-2Rγ mRNAs overlap with that of granzyme D–G mRNAs in the pregnant uterus. Finally, we demonstrate that granzymes D–G are up-regulated by IL-2 and IL-15 in primary cultures containing GMG cells. Taken together, these results suggest that IL-2 and/or IL-15 may regulate GMG cell differentiation in vivo, and that granzymes D–G may have different functions than granzyme A during pregnancy.


During pregnancy in viviparous mammals, the uterus undergoes many changes to nurture and accommodate the developing conceptus. One change that occurs immediately following implantation is the process of decidualization in which there is rapid uterine cell growth and differentiation. This “decidual reaction” is characterized primarily by the differentiation of stromal fibroblasts into decidual cells and by the proliferation and differentiation of granulated metrial gland (GMG) cells (1, 2).

Murine GMG cells belong to the NK cell lineage (3–8), and an analogous cell type, the endometrial granulocyte, has been identified in humans (9–11). In the mouse, GMG cells are localized to the decidua and metrial gland in early to mid-gestation and are confined to the metrial gland by late gestation. GMG cell differentiation begins at about day 7 of gestation and is manifested by the accumulation of cytolytic mediators including perforin and the serine proteases, granzymes A and B, within cytoplasmic granules (7, 12–14). The murine granzymes, A and B, belong to a large family of serine proteases that includes granzymes C, D, E, F, G, and H, metase-1, and tryptase-2 (15). Cytotoxic T cells and NK cells utilize perforin and granzymes to facilitate tumor and virus-infected cell death; however, the precise role of each granzyme has yet to be determined. Recent studies of pregnant transgenic mice deficient in NK and T cells (transgenic strain, TgE26) revealed small numbers of GMG cells, placentae half the size of control mice, and high rates of fetal loss after day 10. Histological analysis of implantation sites between days 10 and 17 revealed abnormalities in the establishment of placental circulation. These results suggest that GMG cell functions may not be important in early pregnancy events such as implantation, but they may be important in placental development (3, 4).

Although the specific functions of uterine GMG cells are unclear, they may regulate trophoblast invasion into the maternal decidua. Indeed, trophoblast killing by murine and human uterine NK cells has been reported (16, 17). Other previously proposed GMG cell functions include 1) lysis of virus-infected cells present in the uterus and placenta, 2) initiation of abortion, 3) destruction of the extracellular matrix and cells at the placental/uterine interface to promote parturition, 4) nutritive functions, and 5) cytokine production (1, 18).

Several cytokines have been implicated in perforin and granzyme gene regulation in both T and NK cells, including IL-2, IL-6, IL-7, IL-12, and IL-15 (19–25). Recently, IL-15 has been shown to up-regulate perforin and granzyme A–B expression in GMG cell explants (26).

Understanding the mechanisms governing GMG cell differentiation during pregnancy will ultimately provide a better understanding of their function and relevance to pregnancy success. In this study, expression of the genes coding for granzymes D, E, F, and G (granzymes D–G) was found to be developmentally regulated in murine GMG cells during pregnancy. Granzymes D–G were shown to be expressed in late gestation, in contrast to the mid-gestational expression of granzyme A. This finding suggests different roles for granzymes D–G compared with granzyme A during pregnancy. Expression of the transcripts coding for the IL-2Rβ and IL-2Rγ was detected in the uterus at the time when granzymes D–G are expressed. Finally, granzymes D–G were shown to be up-regulated by both IL-2 and IL-15 in minced primary cultures containing GMG cells. Therefore, IL-2 and/or IL-15 may regulate GMG cell differentiation in vivo.
Materials and Methods

Animals and tissues

Pregnant CF-1 mice were used. The morning a vaginal plug was observed was designated as day zero of pregnancy. For RNA isolation, uterine tissues removed from days 1 to 6 postcoitum also contained embryonic and placental tissue. Uterine tissues from days 7 to 19 of pregnancy were prepared with the embryos and placentae removed.

Cytokines, Abs, and cDNA clones

Recombinant human IL-15 and recombinant mouse IL-2 were purchased from R&D Systems (Minneapolis, MN), Rabbit polyclonal anti-mouse granulocyte D–G serum was a gift from Dr. Jürg Tschopp (Université de Lausanne, Lausanne, Switzerland). This Ab was prepared against granulocyte D but recognizes all four granulocytes, D–G (27). Horseradish peroxidase conjugated anti-mouse IgG (A) was purchased from Sigma (St. Louis, MO). The mouse IL-2Ra and IL-2Rγ cDNAs were gifts from Dr. Warren Leonard (National Institutes of Health, Bethesda, MD). The mouse granulocyte A cDNA was a gift from Irving Weissman (Stanford University, Stanford, CA). The rat glycerol-aldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was cloned by Fort et al. (28).

Day 11 mouse uterine-specific cDNA library construction

Polyadenylated RNA was obtained from day 11 and day 18 mouse uterine total RNA using Invitrogen Fast Track mRNA isolation reagents (Invitrogen, San Diego, CA). A subtractive library specific for day 11 uterine mRNA was constructed from day 11 and day 18 uterine poly(A+) RNA using the PCR Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) and the T-cloning vector, pT7Blue (Novagen, Madison, WI). The day 11 uterine specific cDNA library contained approximately 10,000 primary recombinants.

cDNA library screen using WSXWS oligonucleotides as probes

The day 11 mouse uterine-specific cDNA library was screened with degenerate oligonucleotides encoding the conserved WSXWS motif of the type 1 cytokine receptors (WSXWS oligo-1: (A/G)CTCCAGGG(A/G)CTCCA and WSXWS oligo-2: (A/G)CTCCATC(A/G)CTCCA) (29). Briefly, the library was plated onto 150-mm Luria-Bertani (LB) agar plates containing 50 μg/ml ampicillin and 15 μg/ml tetracycline at a density of 15,000 colonies/plate. Colonies were replica plated onto 137-mm, 0.45-micron nitrocellulose filters (Microcon Separations, Westboro, MA), grown at 37°C overnight and incubated for an additional 8 h on LB agar plates containing 50 μg/ml chloramphenicol. The bacterial colonies were then lysed and the DNA immobilized by baking at 90°C for 90 min in a vacuum oven. The filters were washed briefly in 6× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7) and prehybridized at 37°C overnight in prehybridization buffer (6× SSC, 2 mg/ml BSA, 2 mg/ml Ficoll, 2 mg/ml polyvinyl pyrrolidone, 100 μM ATP, 10 μM yeast tRNA, 2 mM sodium pyrophosphate, 2 mg/ml salmon sperm DNA, and 0.1% Nonidet P-40 (NP-40)). The WSXWS oligonucleotides (250 pmol) were phosphorilated with T4 polynucleotide kinase (Promega, Madison, WI) and hybridized at 37°C in 1× 10 6 cpm/ml for 28 h in the prehybridization buffer containing 0.1% SDS instead of 0.1% NP-40. The filters were washed once for 10 min at 24°C in 6× SSC, twice for 30 min at 45°C in 6× SSC/0.1% SDS, and twice for 20 min at 45°C in 0.5× SSC/0.1% SDS. The filters were exposed to film for 48 to 96 h at ~70°C with intensifying screens, and then the films were developed. Colonies chosen from the primary screen were subjected to secondary and tertiary screens before pure clones were isolated and sequenced. Sequence analysis was performed with the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package.

cDNA library screen for genes expressed at mid-gestation but not late gestation in the uterus

The day 11 mouse uterine specific cDNA library was plated as in the WSXWS screen. Prehybridization was carried out at 42°C overnight in 4.8× SSC, 48% formamide, 2 mg/ml BSA, 2 mg/ml Ficoll, 2 mg/ml polyvinyl pyrrolidone, 10% dextran sulfate, 0.1% SDS, 10 μg/ml yeast tRNA, and 20 mM Tris, pH 7.6. Day 11 and day 18 mouse uterine cDNAs that had been previously synthesized for subtractive library construction were labeled with [α-32P]dCTP using the Multiprime DNA Labeling System (Amersham, Arlington Heights, IL) to a specific activity of 2 to 4× 10 6 cpm/μg. The unincorporated nucleotides were removed using an STE Select-D G2S spin column (5 Prime—3 Prime, Prime, Boulder, CO). The oligonucleotides were hybridized at 37°C and 1 × 10 7 cpm/ml for 28 h in the prehybridization buffer containing 0.1% SDS instead of 0.1% NP-40. The filters were washed once for 10 min at 24°C in 6× SSC, twice for 30 min at 45°C in 6× SSC/0.1% SDS, and twice for 20 min at 45°C in 0.5× SSC/0.1% SDS. The filters were exposed to film for 48 to 96 h at ~70°C with intensifying screens, and then the films were developed. Colonies chosen from the primary screen were subjected to secondary and tertiary screens before pure clones were isolated and sequenced. Sequence analysis was performed with the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package.

RNA isolation and Northern blot analysis

Total RNA was isolated from frozen tissues using Trizol reagent (GIBCO-BRL—Life Technologies, Grand Island, NY). For Northern blot analysis, 20 to 30 μg of total RNA was separated by electrophoresis on a 1% agarose gel containing formaldehyde and transferred to Zeta Probe GT cationized nylon membranes (Bio-Rad, Hercules, CA). 32P-labeled cDNA probes were synthesized using the Multiprime DNA Labeling System (Amersham) and hybridized at 47°C for 16 to 20 h in 7% SDS, 1% polyethylene glycol (m.w. 15,000–20,000), 40% formamide, 0.1% NP-40, 0.6 M NaCl, 5 mM EDTA, 100 μg/ml yeast tRNA, 100 μg/ml salmon sperm DNA, and 40 mM sodium phosphate, pH 7.4. Following hybridization, the membranes were washed once at 24°C in 0.5× SSC/0.1% SDS and twice at 47°C in 0.2× SSC/0.1% SDS, each for 15 min. RNA transcripts were visualized by autoradiography and quantitated by using ImageQuant software on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Quantitative values for the amount of mRNAs encoding IL-2Rβ, IL-2Rγ, granzymes D–G, and granulyme A were normalized to that obtained for GAPDH in each sample.

Diagnostic RT-PCR analysis of granzymes D–G mRNAs

For RT-PCR, total RNA from day 13 uterus or from the primary minced cell cultures was used. Primers of the following sequences were used: granulyme I primer, TTTAAC/TTA/CCTGTAGAGCA; granulyme II primer, CCTTC/CTG/A/GAGGC/TGAG. cDNA synthesis was performed using Superscript II reverse transcriptase (GIBCO-BRL—Life Technologies, Grand Island, NY). 1 μl of the reaction mixture was used in a 25 μl PCR reaction using one of the following primer sets: granzyme I primer, for primers, 0.5 mM dNTPs, 10 mM MgCl 2 , 0.1% Triton X-100, 50 mM Tris-HCl, pH 9.0. The reaction mix was heated at 94°C for 3 min, and then 0.05 U/μl Taq polymerase (Promega) was added to commence thermal cycling; 30 cycles were performed at 94°C for 45 sec, 56°C for 1 min, and 72°C for 1 min. One final cycle was performed at 72°C for 7 min. In some cases, a trace amount of [α-32P]dCTP (0.1 μCi/μl) was added to the PCR mix to label the products.

Following the PCR, the samples were precipitated in ethanol and re-suspended in water. Restriction analysis was performed with BgIII, BstXI, or HindIII/PoiI (Promega), and the DNA was resolved by electrophoresis on a 1.75% agarose gel containing 0.5 μg/ml ethidium bromide. For 32P-labeled PCR products, the gels were incubated in 10% TCA for 1 h, dried under vacuum, and exposed to PhosphorImager screens. The various DNA fragment intensities were determined using ImageQuant software on the PhosphorImager.

To determine the relative levels of granulyme I and II mRNAs, the specific DNA band intensities from the PhosphorImager were divided by the corresponding restriction fragment size to obtain relative molar equivalents for all bands. The relative molar equivalents were then divided by the sum of all molar equivalents in the gel lane to estimate the fraction of the total represented by each granulyme present.

Primary minced tissue culture

Primary cell cultures were generated similarly to Ye et al. (26). Cultures were generated from day 8 of gestation and contained the entire implantation site. Briefly, the implantation sites (including the embryo, placenta, and uterus) were dissected from the animal and minced into four to six pieces, rinsed in TBS, and cultured in αMEM (GIBCO-BRL—Life Technologies), 2% FCS, 20 mM HEPES (pH 7.4), and 10 mM penicillin/streptomycin. For some experiments IL-15 and/or IL-2 were added to a final concentration of 300 ng/ml, and the cultures were maintained at 37°C for 24 h (26). Following 24-h incubation, samples were frozen in liquid nitrogen and stored at ~70°C prior to RNA isolation.
Western blot analysis

Protein was isolated from tissues by homogenization at 4°C in 0.15 M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 10 μg/ml aprotinin (Sigma), 15 mM sodium phosphate, pH 7.4. After a 30-min incubation at 4°C, the tissue debris was removed by centrifugation at 14,000 × g for 20 min at 4°C. Protein concentrations of the tissue lysates were determined using the Bradford assay. For Western blots, equal amounts of tissue lysate were resolved by SDS-PAGE on 7.5 to 15% gradient gels and transferred to nitrocellulose (NitroBind, Micron Separations). The antigranzyme DEFG serum was used at a dilution of 1:250 and was detected with horseradish peroxidase-conjugated protein A (Sigma) and ECL Western blotting detection reagents (Amersham).

Immunohistochemistry

Entire implantation sites from various days of gestation were fixed in 4% paraformaldehyde for 24 h at 4°C, embedded in paraffin, and cut into 5-μm sections. The sections were incubated with antigranzyme D–G serum (1:500), and the Abs were visualized using an anti-immunoglobulin-peroxidase stain (VectaStain ABC, Vector Laboratories, Burlingame, CA). Sections were then counter stained with periodic acid Schiff’s (PAS) stain and/or hematoxylin.

Results

Granzymes D and G are expressed in the mouse uterus at mid-gestation

To identify genes expressed at mid-gestation but not late gestation in the uterus, a day 11 uterine-specific cDNA library was constructed and screened as follows. The library was probed with radiolabeled cDNAs derived from day 11 and day 18 uterine mRNA, and clones hybridizing to the day 11 uterine probe but not the day 18 probe were selected. In the primary library screen, 26 clones were selected, and 12 of these were analyzed in a secondary screen. Following the secondary screen, 4 of the 12 clones were determined to be differentially expressed in the uterus. Three of these four clones encoded partial cDNAs approximately 600 bp in length that were 98% identical to the previously identified mouse granzyme G cDNA (EMBL/GenBank accession number J02872) (31). The remaining clone encoded a full-length cDNA that was 99.7% identical to mouse granzyme D (EMBL/GenBank accession number J03255) (32).

Temporal expression of granzyme D–G mRNAs in the pregnant mouse uterus

To determine if the genes coding for granzymes D and G were temporally regulated in the uterus during pregnancy, RNA isolated from uteri at various stages throughout gestation was analyzed by Northern blots. The granzyme D cDNA was used as a probe because it would presumably detect both the homologous mRNA and that coding for granzyme G, owing to the 88% identity between these sequences (31). The mRNA transcripts were detected in the uterine samples between days 9 and 16 of pregnancy with peak expression between days 13 and 15 (Fig. 1). In contrast, the uterine expression of granzyme A was observed between days 8 and 13 and peaked on day 9 (Fig. 1). RNA isolated from uterine samples from days 1 through 5 were also screened for granzymes A and D–G. The levels of granzyme mRNAs in these tissues were the same or lower than those determined for uteri isolated from day 7 of gestation.

The nucleotide identities among granzymes D–G range from 84 to 94% (31, 32). Due to these high percentage identities, the granzyme D cDNA probe used in the Northern blot analysis (Fig. 1), even under high stringency conditions, presumably would hybridize to granzymes D, E, F, and G. To determine if all four of these transcripts are expressed during pregnancy, RT-PCR was performed and combined with a diagnostic restriction analysis. Briefly, primers were designed that would amplify granzymes D–G but not granzymes A–C. Restriction analysis, with BstXI, BgIII, or a combination of HindIII/PstI, was used to distinguish the mRNAs encoding granzymes D–G. Figure 2 shows the results of the RT-PCR analysis on day 13 uterine RNA, and it demonstrates that all four granzymes, D, E, F, and G, are expressed. All four granzymes were also detected in day 8 uterine RNA samples (not shown). Therefore, the Northern blot analysis shown in Figure 1 represents the combined gestational profile of granzymes D–G.

Expression of granzymes D–G in uterine GMG cells during pregnancy

Cytolytic mediators such as perforin, granzyme A, and granzyme B have been localized to uterine GMG cell granules during pregnancy (7, 12–14). Examination of implantation sites by immunohistochemistry with an antigranzyme D–G serum, revealed that granzymes D–G are also expressed in GMG cells (Fig. 3). Figure 3C shows intense granzyme immunoreactivity on day 15 of pregnancy in a large population of decidual cells but little staining in the placenta. The intense staining was also observed in the metrial
gland located just above the decidua (metrial gland, not shown). Traditionally, GMG cells have been identified by their morphology and granular PAS staining (33, 34). The granzymes D–G stain was colocalized with the PAS stain in the sections analyzed (Fig. 3, B–D), thus demonstrating that these proteins are expressed in the GMG cells. In addition, the granzymes D–G protein expression

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FIGURE 2. Granzymes D, E, F, and G mRNAs are expressed in the murine uterus during pregnancy. Day 13 uterine RNA was analyzed by RT-PCR, and granzymes D–G were identified by distinct restriction fragments created by BglII, BstXI, or HindIII/PstI digestion of the PCR products. Left, An agarose gel showing the uncut and cut PCR products. A 100-bp ladder is shown with the smallest visible band being 100 bp. Right, Diagrammatic representation of granzyme D–G cDNAs showing the regions that were amplified by PCR and the sizes of the DNA fragments expected after restriction analysis. All fragments predicted from the restriction analysis were found in the agarose gel.

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FIGURE 3. Distribution of granzymes D–G in uterine GMG cells during pregnancy. Implantation site sections from day 15 of gestation: (A) negative control, immunostained but minus primary Ab; (B) PAS stain; (C) antigranzyme D–G antiserum; and (D) PAS stain and antigranzyme D–G antiserum. The tip of the arrow in B denotes the interface between the decidua (above) and the placenta (below). GMG cells in the decidua were identified by their granular PAS staining (B). The double stain with PAS and antigranzyme D–G antiserum confirmed that granzymes D–G are expressed in GMG cells (D). Final magnification of photographs, ×65.
IL-15 receptor consists of IL-2Rβ affinity trimeric receptor (IL-2Rg). Granzymes D–G expression in GMG cells. IL-2 signals via a high affinity trimeric receptor (IL-2Rα, IL-2Rβ, and IL-2Rγ) and an intermediate affinity dimeric receptor (IL-2Rβ and IL-2Rγ). The IL-15 receptor consists of IL-2Rβ and IL-2Rγ with an additional subunit that has been demonstrated to exist in the uterus (26).

Expression of IL-2Rβ and IL-2Rγ in the uterus during gestation
A second library screen was performed to isolate cDNAs encoding type I cytokine receptors expressed in the mid-gestational uterus. In this screen, the day 11 uterine-specific library was probed with oligonucleotides encoding the conserved WSXWS motif found in this family of receptors (35). Two different oligonucleotides were used, WSXWS-1 and WSXWS-2 (see Materials and Methods). Using the WSXWS-2 oligonucleotide, a partial IL-2Rβ (~130 bp) cDNA was isolated. This clone was 99% identical to the previously identified mouse cDNA sequence (EMBL/GenBank accession number M28052).

The expression of IL-2Rβ mRNA was examined in uterine tissues over the course of gestation and determined to be temporally regulated (Fig. 5). The IL-2Rβ mRNA was detected between days 8 and 15 and reached its highest level on day 9. In addition, the period of IL-2Rβ mRNA expression overlapped with the expression of granzyme A and granzymes D–G mRNA (Figs. 1 and 5).

The concomitant uterine IL-2Rβ and granzymes D–G expression prompted us to determine if IL-15 and/or IL-2 could regulate granzymes D–G expression in GMG cells. IL-2 signals via a high affinity trimeric receptor (IL-2Rα, IL-2Rβ, and IL-2Rγ) and an intermediate affinity dimeric receptor (IL-2Rβ and IL-2Rγ). The IL-15 receptor consists of IL-2Rβ and IL-2Rγ with an additional subunit that has been demonstrated to exist in the uterus (26).

IL-2 and IL-15 induction of granzyme D–G expression in primary cultures containing GMG cells
The fact that the receptor subunits utilized in IL-2 and IL-15 signaling are present in the uterus at the time when granzymes D–G are expressed suggested that IL-2 and/or IL-15 may regulate granzyme D–G expression in GMG cells. To examine this possibility, primary minced implantation sites from day 8 of gestation were cultured with IL-2 and/or IL-15, and the amount of granzyme D–G mRNAs present after 24 h was determined. A 2- to 5-fold increase in granzyme D–G mRNAs was observed in the primary cultures incubated with IL-15, whereas cultures treated with IL-2 showed a 3- to 6-fold increase (Fig. 6). The combination of both cytokines resulted in a stimulation that was approximately equal to that obtained with either cytokine alone (not shown). To eliminate possible influences of cells in the cultures other than the decidual cells, the effects of IL-2 and IL-15 were also tested in cultures containing the decidua alone. The effects of IL-2 and IL-15 on granzyme D–G mRNAs were similar in the cultures containing only decidual cells.
compared to those containing entire implantation sites. In an experiment in which the response to IL-15 of complete implantation sites and isolated decidua were tested in triplicates, the former showed a 3.6-fold stimulation over the control and the latter showed a 5-fold stimulation over the control value.

To determine if granzymes D–G are differentially regulated by IL-15 and IL-2, the RNA from the primary cultures was analyzed by RT-PCR and diagnostic restriction analysis as outlined in Figure 2. When the relative amounts of the various granzymes in each sample were compared between stimulated and unstimulated samples, there was no significant difference in the relative amounts of any of the granzyme mRNAs in response to either IL-2 or IL-15 (Fig. 7). Thus, we conclude that IL-2 and IL-15 equally increase the expression of granzyme D–G mRNAs.

Discussion

In this report, the genes encoding granzymes D–G were shown to be temporally regulated in the mouse uterus during pregnancy. Furthermore, granzymes D–G were localized to uterine GMG cells between days 9 and 17. Perforin and granzymes A and B have also been localized to GMG cells (7, 12–14) with peak mRNA expression on day 9 of pregnancy (26). Our results show that the granzyme A transcript is expressed between days 7 and 13 with a sharp peak at day 9. In contrast, granzyme D–G mRNAs expression occurs between days 8 and 16, with a broad peak between days 13 and 15. This finding supports the hypothesis that granzymes D–G may have different roles than granzymes A and B during pregnancy. In addition, this is the first report showing the expression of granzyme F in NK cells, as this granzyme was previously reported to have restricted expression in the CD4−CD8+ subset of peripheral T cells (36).

Many investigators have proposed that GMG cells may lyse placental trophoblasts invading the maternal decidua. Controlling the extent of trophoblast invasion is extremely important to the maintenance of pregnancy, because in humans diminished invasion may result in problems such as pre-eclampsia and still birth, whereas extensive invasion may cause maternal death due to uterine rupture (37). The granzyme A and B expression patterns are well timed with respect to the period of trophoblast invasion, and perhaps they play a role in these events. GMG cells only have lytic activity between days 6.5 and 9.5 of gestation (38), and this lytic activity is coincident with granzyme A and B expression and with the period of trophoblast invasion.

The late gestational expression of granzymes D–G suggests that these serine proteases may not be regulators of trophoblast invasion. Parturition, which requires considerable tissue remodeling, occurs between 19 and 21 days of gestation in the mouse. Although there is no experimental evidence supporting regulated granzyme secretion from GMG cells, Croy et al. (39) proposed that granzymes may be released in late gestation to eliminate the extracellular matrix and cells at the uterine/placental interface to promote parturition. Experimental evidence in support of this hypothesis was recently demonstrated. Delgado et al. (40) showed that GMG cells began to undergo degenerative changes from day 12 through parturition. Furthermore, degenerating GMG cells rupture and release their granule contents in late gestation (39, 40).

In this study, granzyme D–G proteins were detected between days 9 and 17 in the uterus. Mesometrial decidual regression begins at about day 10 and continues until birth (2, 41). Although they are generally believed to be involved in cell-mediated cytotoxicity, there is some evidence that granzymes can also participate in other events (42). It is intriguing to consider that granzymes D–G released from degenerating GMG cells in late gestation may prepare the fetomaternal interface for parturition.

The mechanisms governing GMG cell differentiation during pregnancy are not well understood, and determining the signaling pathways required for their differentiation may ultimately lead to a better functional understanding of these cells. In this study, Northern blot analysis of uterine tissues revealed that the IL-2Rβ mRNA is temporally regulated during pregnancy. Although mouse uterine IL-2Rβ expression has been reported in the past (26, 43), its quantitative mRNA profile over the course of gestation has not previously been reported. The IL-2Rβ subunit was shown to be expressed between days 8 and 16, with peak expression on day 9. This expression pattern, overlapping with that of granzymes D–G, prompted us to investigate whether IL-2 and/or IL-15, the only cytokines known to bind the IL-2Rβ, regulate granzyme D–G expression and therefore GMG cell differentiation. Because IL-2 and IL-15 signaling occurs through several heteromeric receptors, uterine tissues from various days of gestation were analyzed for the
additional receptors that might be involved, the IL-2Rα and IL-2Rγ. By Northern blot analysis, the IL-2Rα mRNA was undetectable throughout gestation. In agreement with this finding, Ye et al. (26) also did not detect the IL-2Rα mRNA by RT-PCR. However, low levels of IL-2Rα have been reported in the murine and human uterus by other investigators (43–45). The IL-2Rγ mRNA was present throughout gestation and increased at mid-gestation following a similar pattern to what was observed for the IL-2Rβ mRNA. Together, these experiments provide evidence that intermediate affinity IL-2 receptors (IL-2Rβ/IL-2Rγ) are present in the mouse uterus at mid-gestation.

Ye et al. (26) reported mid-gestational expression of mouse IL-15Rα in the uterus, which they detected by RT-PCR analysis. Combined with our results of the IL-2Rβ and IL-2Rγ mRNA expression, these results suggest that an IL-15R composed of the IL-15Rα, IL-2Rβ, and IL-2Rγ may exist in the uterus at mid-gestation.

To determine whether IL-15 and/or IL-2 regulate granzyme D–G expression in GMG cells, primary miniced tissue cultures that contained GMG cells were treated with both of these cytokines individually or together. GMG cells represent the predominant immune effector cell population in the rodent uterus during pregnancy (38). Therefore, any effect on granzyme D–G expression in these primary miniced cultures are likely to be a response of GMG cells. IL-15 and IL-2 stimulated granzyme D–G expression to similar levels in day 8 primary implantation cultures. The combination of IL-2 and IL-15 was not synergistic, suggesting that IL-2 and IL-15 may use the same signal transduction pathways in GMG cells.

In addition to Northern blot analysis, diagnostic RT-PCR was performed on RNA isolated from the primary cultures to determine if granzymes D–G were differentially regulated by IL-2 and IL-15. The analysis showed no change in the relative proportions of individual granzyme mRNAs after addition of IL-15 or IL-2. This suggests that the 2- to 6-fold increase in granzyme D–G mRNAs observed on Northern blots was due to the cumulative increase in the mRNAs encoding all four granzymes.

Ultimately, the in vivo regulation of perforin, granzymes A and B, and granzymes D–G gene expression will depend on the presence of IL-2 and IL-15 in the local GMG cell environment. Expression of IL-2 mRNA has been reported in the human and murine placenta (46–48) and in human decidual and T cells (49, 50). Interestingly, the placenta of mice that are prone to high rates of spontaneous fetal resorptions contain elevated IL-2 mRNA (47). This is in agreement with other reports showing that elevated IL-2 levels have deleterious effects on pregnancy (51). Our results suggest that uterine GMG cells may be IL-2 targets in vivo. IL-2 is widely recognized as a regulator of T and NK cell activation and most activated T and NK cells express cytolytic mediators. In analogy, elevated IL-2 expression may aberrantly activate GMG cells to a highly lytic state resulting in extensive cell lysis and abortion. IL-15 mRNA expression has also been reported in the placenta and uterus during pregnancy (26, 52). While IL-2 is expressed mainly in T cells, IL-15 has been detected in a wide variety of cells and tissues (52). This recently identified cytokine has been shown to function similarly to IL-2 in many instances (53, 54), whereas it is expressed in many different cell types and probably has functions outside the immune system. For example, IL-15 can act as an anabolic agent in muscle cells (55). Therefore, although our studies and the results of others show that IL-15 may regulate GMG cell differentiation (26), it may have additional effects on GMG cells during pregnancy as well as distinct effects on specific granzymes.

Although the in vivo regulators of GMG cell differentiation remain to be elucidated, the primary cultures in this study provide strong evidence that IL-2 and/or IL-15 may be involved. In addition, the distinct expression patterns of granzyme A and granzymes D–G provide further evidence that granzymes may have several functions throughout pregnancy. Ultimately, determining granzyme functions during pregnancy may provide a better understanding of normal placentation, uterine tissue remodeling, and fetal/maternal interactions in general.

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References


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