Oocyte-Specific Expression of Growth/Differentiation Factor-9

Sharon A. McGrath, Aurora F. Esqueda, and Se-Jin Lee

Department of Molecular Biology and Genetics
Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

Growth/differentiation factor-9 (GDF-9) is a previously described member of the transforming growth factor- β superfamily expressed specifically in the ovary in adult mice. Using *in situ* hybridization methods, we have localized the expression of GDF-9 messenger RNA (mRNA) exclusively to oocytes. GDF-9 mRNA was detected in oocytes at all stages of follicular development, except in primordial follicles, in both neonatal and adult ovaries. GDF-9 mRNA continued to be expressed in oocytes after ovulation, but disappeared by 1.5 days after fertilization. Based on Western analysis of ovarian extracts using antibodies raised against recombinant GDF-9 protein, GDF-9 mRNA expressed by oocytes appears to be translated. A human homolog of GDF-9 was isolated from a complementary DNA library prepared from adult ovary mRNA. The predicted human protein is 90% identical to murine GDF-9 in the mature portion of the molecule. These results are significant because no other growth factor-like molecules have been shown to be expressed specifically by oocytes and, together with results of previous studies, suggest that ovarian development and function are regulated by factors produced by both oocytes and support cells of the ovary. (Molecular Endocrinology 9: 131–136, 1995)

INTRODUCTION

A large number of growth and differentiation factors believed to play essential roles in regulating development have been found to be structurally related to transforming growth factor- β (TGF β). Among the known members of the TGF β superfamily, for example, are decapentaplegic, which is necessary for proper dorsal/ventral axis formation and development of the imaginal disks in *Drosophila* (1); Vg-1 (2, 3) and the activins (4–7), which can induce mesoderm formation in *Xenopus* animal caps; nodal, which appears to be essential for mesoderm formation in mice (8); dorsalin-1, which can regulate neural crest cell migration and motor neuron differentiation in chick neural

plate explant cultures (9); Mullerian inhibiting substance, which is essential for normal male sex development in mammals (10–12); the bone morphogenetic proteins, which are capable of inducing *de novo* cartilage and bone formation and appear to be essential for normal skeletal development during mammalian embryogenesis (13–18); and glial cell-derived neurotrophic factor, which can promote the survival of mid-brain dopaminergic neurons (19).

The biologically active forms of these secreted factors are believed to be generated by proteolytic cleavage from a larger precursor protein. For the most part, it is only in the mature carboxy-terminal fragments that the different family members are related with respect to their amino acid sequence. To identify additional factors that may play important roles in regulating cell differentiation processes during development, we have isolated novel TGF β family members using degenerate oligonucleotides corresponding to conserved regions among the known family members. We previously reported the identification of a distantly related family member [designated growth/differentiation factor-9 (GDF-9)] expressed specifically in the ovary in adult mice (20). Here we report that GDF-9 is expressed exclusively by oocytes. The oocyte specificity of GDF-9 is unique among known growth factor-like molecules and suggests that oocyte development in mammals is regulated by a complex interplay of factors elaborated by both oocytes and support cells.

RESULTS

Expression of GDF-9 Messenger RNA (mRNA) in Oocytes

By Northern analysis, we previously detected GDF-9 mRNA expression only in the ovary among a large number of adult tissues surveyed (20). To localize the expression of GDF-9 within the ovary, we carried out *in situ* hybridization experiments using GDF-9 RNA probes on sections of ovaries prepared from randomly cycling adult mice. Figure 1 shows representative fields of adjacent sections hybridized with either antisense or sense RNA probes transcribed *in vitro* from plasmids containing portions of the murine GDF-9 complementary DNA (cDNA) sequence. The antisense

Fig. 1. Localization of GDF-9 mRNA in Adult Ovary Sections

Adjacent sections of ovaries prepared from randomly cycling mice were hybridized with either antisense (*left panel*) or sense (*right panel*) GDF-9 riboprobes, dipped in photographic emulsion, developed, and stained with hematoxylin and eosin. A primordial follicle negative for GDF-9 expression is noted by an *arrow*.

probe detected GDF-9 mRNA present exclusively in oocytes. No hybridization was seen with a control sense probe, and no hybridization was detected in any other ovarian cell type, including thecal and granulosa cells. GDF-9 mRNA was detected in oocytes at all stages of follicular development, except for those present in primordial follicles. Although the intensity of hybridization varied somewhat among different oocytes, there was no obvious correlation between the level of GDF-9 mRNA expression and the stage of follicular development.

Expression of GDF-9 mRNA during Oocyte Development

To further characterize the expression of GDF-9 in oocytes, we carried out *in situ* hybridization experiments on sections of ovaries isolated at various developmental stages. The earliest stage of ovarian development that we examined were ovaries taken from day 17.5 postcoital (pc) embryos. At this stage, the oocytes were small and present in primordial follicles, which were characterized by a single layer of squamous follicular cells. By *in situ* hybridization, we were unable to detect the expression of GDF-9 mRNA in these fetal oocytes (data not shown).

In contrast, GDF-9 mRNA was clearly present in postnatal oocytes. By postnatal day 4, many of the follicles were characterized by the presence of an enlarged oocyte surrounded by more cuboidal or columnar follicular cells. As shown in Fig. 2A, oocytes present in these primary follicles all showed GDF-9 mRNA expression, whereas oocytes present in primordial follicles were all negative. By postnatal day 8, intense hybridization was seen in oocytes in primary follicles (Fig. 2B).

We also examined the expression of GDF-9 mRNA in oocytes after ovulation. As shown in Fig. 3A, GDF-9 mRNA continued to be expressed by oocytes that had been released into the oviduct. However, GDF-9 mRNA expression appeared to be lost rapidly after

Fig. 2. Expression of GDF-9 in Neonatal Ovaries

Sections of ovaries isolated from postnatal day 4 (A) or postnatal day 8 (B) mice were hybridized with either antisense (*left panels*) or sense (*right panels*) GDF-9 riboprobes and analyzed as described in Fig. 1.

fertilization. By day 0.5 pc, GDF-9 mRNA was detected in some, but not all, embryos (Fig. 3B), and by day 1.5 pc, GDF-9 mRNA expression was no longer detected (data not shown). In day 0.5 pc embryos in which GDF-9 mRNA was detected, the intensity of

Fig. 3. Expression of GDF-9 in Unfertilized and Fertilized Oocytes

A, Sections of oviducts isolated from unmated mice were hybridized with either antisense (*left panel*) or sense (*right panel*) GDF-9 riboprobes and analyzed as described in Fig. 1. B, Sections of oviducts isolated from day 0.5 pc females were hybridized with antisense GDF-9 riboprobes and, after autoradiography, photographed under either brightfield (*left panel*) or darkfield (*right panel*) illumination.

hybridization appeared to be highest at the periphery of the cell (Fig. 3B). Although only a single embryo is shown, the ring-like pattern of hybridization was also seen in two other embryos at this stage (data not shown). This pattern of hybridization was less pronounced in unfertilized oocytes and was never seen in oocytes before ovulation or with a control sense probe in oocytes or embryos at any stage of development.

Detection of GDF-9 Protein in Adult Ovary

To determine whether GDF-9 mRNA expressed in the ovary is translated into protein, we raised antisera against GDF-9 protein. To avoid possible cross-reactivity of these antibodies with related proteins, we prepared antibodies directed against the pro region of GDF-9, which showed no obvious sequence homology to the pro regions of other known family members. Specifically, we expressed the portion of GDF-9 spanning amino acids 30–295 in bacteria and immunized rabbits with gel-purified protein. The presence of GDF-9-reactive antibodies in the serum of these rabbits was

verified by Western analysis of bacterially expressed protein fragments (data not shown).

As shown in Fig. 4, Western analysis using these anti-GDF-9 antibodies detected a protein with an approximate mol wt of 37,000 in extracts prepared from adult ovary. This band was not seen in a variety of other tissues examined. Based on the cDNA sequence (20), the full-length GDF-9 protein was predicted to have a mol wt of approximately 49,600. After proteolytic cleavage following the signal sequence and at the putative processing site at amino acids 303–306, the pro region was predicted to have a mol wt of approximately 31,000. We presume that the difference between the apparent mol wt and the predicted mol wt represents the presence of post-translational modifications, such as glycosylation at one or more of the three potential *N*-glycosylation sites present in the pro region.

Isolation of Human GDF-9

Because GDF-9 was originally identified in mice, which are multiparous, we sought to determine whether GDF-9 homologs are also present in uniparous species. In particular, we prepared a cDNA library from RNA isolated from human ovaries and screened this library using a murine GDF-9 cDNA probe. From 7.5 million primary recombinant phages screened, we isolated 12 hybridizing clones and determined the entire nucleotide sequence of the longest insert. The 1774-basepair sequence contained a single long open reading frame encoding a protein highly related to murine GDF-9 (Fig. 5). Like the predicted murine GDF-9 amino acid sequence, the predicted human sequence contained a hydrophobic stretch of amino acids at the N-terminus suggestive of a signal sequence for secretion, a putative RXXR cleavage site 135 amino acids from the C-terminus, and a C-terminal region that showed significant homology to other members of the TGF β superfamily. As is characteristic

Fig. 4. Expression of GDF-9 Protein

Extracts prepared from various adult tissues were electrophoresed on SDS-polyacrylamide gels and either stained with Coomassie blue or transferred to nitrocellulose and probed with anti-GDF-9 (immune) or preimmune sera. Numbers at the *left* indicate the mobilities of the mol wt standards.

Fig. 5. Comparison of the Predicted Amino Acid Sequences of Murine (*Top Lines*) and Human (*Bottom Lines*) GDF-9

Numbers indicate amino acid positions relative to the N-termini. The predicted proteolytic processing sites are boxed. The conserved cysteine residues in the C-terminal regions are shaded.

of other members of the TGF β superfamily, the homology between murine and human GDF-9 was significantly higher in the mature C-terminal portion (~90% amino acid sequence identity) than in the pro region (~60% amino acid sequence identity) of the molecule.

DISCUSSION

GDF-9 was originally identified by polymerase chain reaction using degenerate oligonucleotides corresponding to conserved regions among known members of the TGF β superfamily (20). Although GDF-9 is clearly a new member of the TGF β superfamily, GDF-9 is only distantly related to other family members. By Northern analysis, GDF-9 mRNA was detected only in the ovary among a large number of adult tissues surveyed. In this report, we describe the localization of GDF-9 mRNA exclusively to oocytes in both neonatal and adult ovaries. The onset of GDF-9 expression during ovarian development appears to coincide with the formation of primary follicles. GDF-9 mRNA continues to be expressed by oocytes throughout follicular development as well as after ovulation, but disappears by day 1.5 after fertilization.

Other growth factor-like molecules are known to be expressed in the ovary and are believed to influence

ovarian development and function (for review, see Ref. 21). These include members of the TGF β superfamily, such as Mullerian inhibiting substance (12, 22–24), Vgr-1 (25), inhibin and activin subunits, and the TGF β s themselves (21), as well as members of other growth factor families, such as steel factor (26), epidermal growth factor, TGF α , tumor necrosis factor- α , insulin, insulin-like growth factors, basic fibroblast growth factor, and interleukin-1 (21). However, the expression pattern of GDF-9 is unique compared with those of other growth factor-like molecules in its apparent specificity for the ovary and, in particular, for the oocyte. In fact, to our knowledge, Vgr-1 is the only other growth factor-like molecule that has been demonstrated to be expressed by oocytes, and unlike GDF-9, Vgr-1 is also expressed by other cell types within the ovary as well as in other tissues (25, 27). The oocyte specificity of GDF-9 suggests that oocyte maturation involves not only growth factors produced by support cells of the ovary, but also factors secreted by the oocyte itself.

Although the biological function of GDF-9 is not yet known, the expression pattern of GDF-9 suggests several possible regulatory roles for GDF-9. For example, GDF-9 may play an autocrine role in regulating the maturation of the oocyte. In this regard, at least two unidentified factors have been detected in follicular fluid that can maintain oocytes in meiotic arrest (28, 29), although the apparent mol wt of these factors do not correspond to that predicted for GDF-9. Alternatively, GDF-9 may play a paracrine role. One possibility, for example, is that GDF-9 may play a role in regulating the proliferation and/or differentiation of granulosa cells. Although there have been many hypotheses put forth regarding the role of oocyte-derived factors in regulating follicle formation, such factors have not yet been identified or characterized. Another potential paracrine role for GDF-9 might be in regulating sperm-egg interactions. In sea urchins, it is known, for example, that oocytes produce factors that are chemotactic for sperm (30), and there are some reports that such factors may also exist in mammals (31, 32). Finally, it is possible that GDF-9 may be involved in regulating early embryonic development. We do not believe, however, that GDF-9 is solely a stored maternal mRNA to be used during early embryogenesis, because we have been able to detect the presence of GDF-9 protein in adult ovary. Nevertheless, the relatively long window of GDF-9 expression during oocyte development suggests that perhaps GDF-9 may play multiple regulatory roles.

Whatever role(s) GDF-9 may play in regulating ovarian development and function, the function of GDF-9 does not appear to be unique to animals with multiple ovulatory potential. In particular, we have identified a human gene that encodes a protein that is 90% identical to murine GDF-9 in the predicted mature portion of the molecule. The high degree of sequence conservation between murine and human GDF-9 suggests

that GDF-9 carries out an important function in both multiparous and uniparous species.

MATERIALS AND METHODS

In Situ Hybridizations

For RNA localization studies, all tissue samples were taken from CD-1 mice (Charles River, Wilmington, MA). All animal studies were conducted in accord with the principles and procedures outlined in the Guidelines for Care and Use of Experimental Animals. Timed pregnancies were obtained from random matings, and the beginning of day 0 was considered to be the midpoint of the dark cycle preceding the morning on which the vaginal plug was noted. Ovaries or oviducts were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. *In situ* hybridizations were carried out as previously described (33). ³⁵S-Labeled riboprobes were transcribed from GDF-9 cDNA subclones representing nucleotides 1–903 or 901–1644 (20). The two antisense (or sense) probes from these subclones were mixed together for hybridization. Autoradiography was carried out using NTB-3 photographic emulsion (Eastman Kodak, Rochester, NY). Exposure times ranged from 7–10 days.

Detection of GDF-9 Protein

For antibody production, the GDF-9 coding sequence spanning amino acids 30–295 was cloned into the pET3 expression vector (kindly provided by F. W. Studier), and the resulting plasmid was transformed into BL21 (DE3) cells. Cells were induced with isopropyl β -D-thiogalactoside, and GDF-9 protein was gel purified from total cell extracts. All immunization procedures were carried out by Spring Valley Lab (Sykesville, MD). Tissue extracts were prepared by homogenization in sample buffer. Western analysis was carried out as previously described (34), except that 5% nonfat dried milk was used as the blocking agent. Antisera were used at a dilution of 1:1000.

Isolation of Human GDF-9

Human ovaries were obtained from the International Institute for the Advancement of Medicine (Exton, PA). RNA isolation, cDNA library construction, and isolation and characterization of cDNA clones were carried out as described previously (35).

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Address requests for reprints to: Dr. Se-Jin Lee, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205.

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