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OVARIAN EPIDERMAL GROWTH FACTOR-LIKE ACTIVITY. CONCENTRATIONS IN PORCINE FOLLICULAR FLUID DURING FOLLICULAR ENLARGEMENT

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SUMMARY: **Numerous data indicate that epidermal growth factor has important effects on cultured granulosa cells. However, most of the few attempts to detect epidermal growth factor in ovarian tissue have been unrevealing. In this study, ovarian epidermal growth factor-like activity was easily detected by a radioreceptor assay based on the A431 cell line but not by an immunoassay for mouse epidermal growth factor. The concentration of this activity in follicular fluid from small porcine ovarian follicles was higher than that in fluid from medium or large follicles or serum ($p<0.01$), but lower than that in salivary gland extracts. Receptor-active epidermal growth factor-like peptides could function as local ovarian regulators.**

Despite the repeated demonstration of epidermal growth factor (EGF) effects on ovarian granulosa cells (reviewed in Ref. 1), evidence concerning the presence of EGF in the ovary is limited. Ovarian tissue has been included in several tissue surveys for localization of EGF by immunochemical techniques (2-4), and more recently by hybridization studies with cDNA probes for EGF precursor mRNA (4). One study found EGF immunoreactivity in a few ovarian follicles (3), but most reports suggest an absence of such activity in the ovary (2,4). The precursor mRNA for EGF is evidently present in the ovary, albeit at low levels (4). On the other hand, cultured Sertoli cells, the male homologue of granulosa cells, secrete a peptide growth factor which is recognized by EGF receptors (5) and which could constitute the ligand for gonadal EGF effects *in vivo*. Using the A431 cell line as the basis for a radioreceptor assay (6), we have analyzed the concentrations of EGF-like activity in the fluid from porcine ovarian follicles at various stages of development. These analyses have shown substantial EGF-like activity in the porcine ovary and suggested local regulation of its concentration.

Abbreviation used: EGF - epidermal growth factor.

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MATERIALS AND METHODS

Biological Material: Porcine ovaries were collected at a local abattoir and divided into those from cycling animals (with luteal tissue) and those from presumptively immature animals (without luteal tissue). Follicular fluid from each type of ovary was aspirated separately and fluid from small (1-2 mm), medium (3-6 mm), and large (>6 mm) follicles collected and pooled as previously described (7). Samples of fluid from small follicles were pooled from 50-100 ovaries; samples from medium and large follicles derived from 6-10 ovaries. After the granulosa cells were removed by centrifugation, follicular fluid was placed in plastic tubes containing a mixture of protease inhibitors, final concentrations: 10 ng/ml soybean trypsin inhibitor, 10 ng/ml leupeptin, 1 ng/ml pepstatin A, 4 ng/ml aproteinin, and 75 ng/ml phenylmethylsulfonylfluoride. All protease inhibitors were from GIBCO Laboratories (Grand Island, New York). Serum and plasma samples were obtained from gilts at the abattoir on the same day as the ovarian samples and processed identically to follicular fluid. Porcine plasma was prepared from whole porcine blood (anticoagulated with citrate) by centrifugation, initially at 300 X g to remove red blood cells, and then at 20,000 X g for 20 min to remove platelets. The same anticoagulant, added to serum, did not influence assay results. Finally, an extract of porcine submaxillary glands was prepared by homogenization of previously frozen glands with two volumes of Tris-HCl (10 nM, pH 7.4) with a Polytron homogenizer (Brinkman Instruments, Westbury, NY), followed by centrifugation at 15,000 X g for 30 min at 4C.

Radioreceptor Assay for EGF-Like Activity: Initially, frozen samples of serum and follicular fluid were assayed in the laboratory of Dr. S.D. Holmes by published methods (5). These results were systematically lower, but otherwise in general agreement with those reported here, which reflect analyses performed in Hershey on freshly prepared samples using similar methods. Briefly, 5-7 X 10^4 A431 cells (American type culture collection, Rockville, MD) were cultured in 24-well Falcon Multiwell plates (Becton-Dickinson, Oxnard, CA) in 1 ml Dulbecco's modified Eagles medium with 10% fetal bovine serum. Prior to assay the monolayers were fixed in 10% formalin and washed with assay buffer [Dulbecco's phosphate buffered saline (GIBCO Laboratories), pH 7.2, containing 0.1% bovine serum albumin]. Samples, standards and tracer (0.2 ng) in a total volume of 0.5 ml were added to the multiwells, and incubated for 90 min at 37C. Then the monolayers were washed 3 times with assay buffer and dissolved in 1 N NaOH. The bound radioactivity was determined by gamma spectroscopy. Mouse EGF (Collaborative Research, Bedford, MA) was used for standard and radioligand. The sensitivity of the assay was $\leq$ 0.4 ng/well EGF and the coefficient of variation was 10% within assays and 15% between assays.

EGF Immunoassay: The immunoassay method, adapted from Hirata and Orth (8), used mouse EGF for standard and radioligand as described for the receptor assay. In addition, rabbit anti-mouse EGF antisera (lot no. 861191, Collaborative Research) was used in a final dilution of 1/75,000. Standards, tracer and samples were incubated with antibody in a total volume of 0.5 ml overnight at 4C. Thereafter, goat anti-rabbit serum and rabbit serum were added. The immunoprecipitate was collected by centrifugation and the radioactivity determined by gamma spectroscopy. The sensitivity of this assay was $\leq$ 0.2 ng/tube.

Statistics: The differences between means were analyzed by one way analysis of variance and the Newman-Keuls procedure.

RESULTS

In both the radioreceptor and immunoassay procedures recovery of mouse EGF added to samples was complete. In addition, proteolytic degradation of

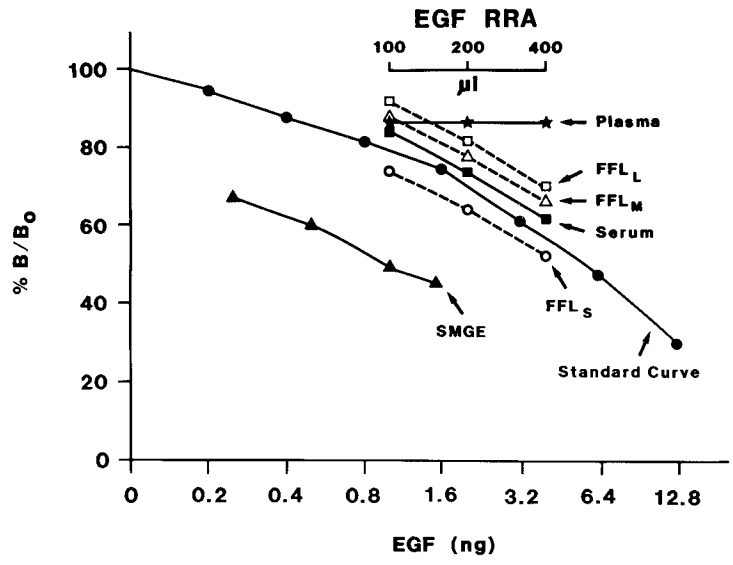

Fig. 1. Dose-dependent displacement of [^{125}I]-iodo-EGF by follicular fluid (FFL), plasma, serum and submaxillary gland extract (SMGE) in EGF radioreceptor assay (RRA). Subscripts following FFL (S,M,L) designate origin from small, medium or large follicles. Values represent radioactivity bound, expressed as a percent of initial binding (B_0).

[^{125}I-iodo-EGF tracer was evaluated by precipitation of tracer with 5% trichloroacetic acid before and after incubation with follicular fluid or serum. In both instances, the radioligand was stable by this criteria. On the other hand, the active principle detected by receptor assay in follicular fluid was found to be labile in storage. The use of proteolytic enzyme inhibitors resulted in an average 50% increase in the estimates of EGF-like activity in stored samples. To minimize the influence of these losses, we are reporting values derived from fresh samples, analyzed as soon as possible after collection and treated in addition with proteolytic enzyme inhibitors.

As depicted in Figure 1 the EGF-like activity in porcine follicular fluid, submaxillary gland and serum, but not plasma, displaced [^{125}I]-iodo-EGF from the receptors on A431 cells in a dose-dependent manner, parallel to that observed with mouse EGF. In contrast, the activity present in follicular fluid, serum, plasma and submaxillary gland (data not shown) failed to displace tracer in a radioimmunoassay employing an antiserum to mouse EGF, despite comparable sensitivity for mouse EGF in the receptor and immunoassay procedures. The estimates of EGF-like activity by radioreceptor assay in various samples of follicular fluid and serum are shown in Fig. 2. Follicular fluid from small follicles from immature animals contained the highest concentration of EGF-like activity (equivalent to 13.6±1.0 ng/ml mouse EGF). This value was significantly ($p<0.01$) different

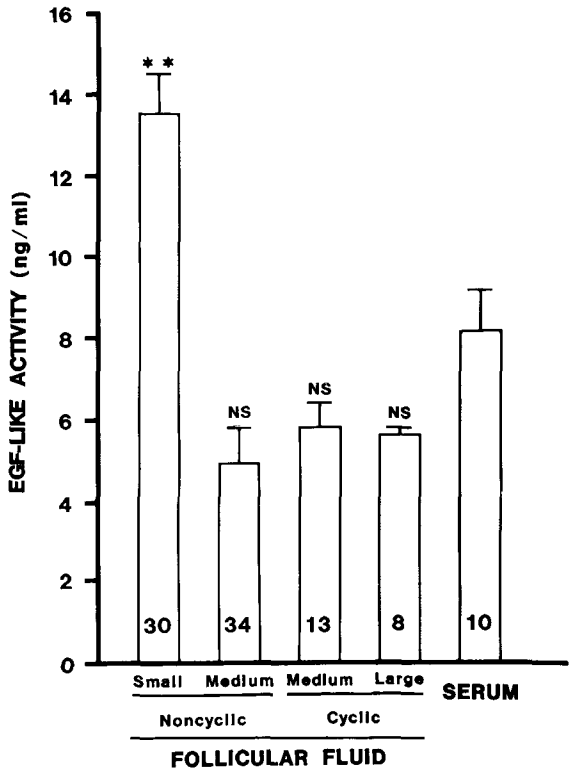

Fig. 2. Concentration of EGF-like activity in follicular fluid and serum. EGF-like activity in different sizes of follicles and classes of ovaries as well as in porcine serum was determined by an EGF radioreceptor assay. The number of samples examined is shown within the bars. Estimates of activity were extrapolated from a standard curve employing mouse EGF, and the values given are mean ± SE (**, different from all other bars, $p<0.01$; NS, not different from serum, $p>0.1$).

from the values in medium-sized follicles from both cycling and non-cycling animals) or in large follicles or serum. In contrast, the concentrations of EGF-like activity in serum and follicular fluid from medium and large follicles irrespective of the type of ovary were not significantly different ($p>0.1$).

DISCUSSION

To our knowledge, these data represent the first systematic measurements of ovarian EGF-like activity by radioreceptor assay. The concentrations determined by this technique are easily measurable and well within the range of those expected to influence granulosa cell replication and differentiated function (see below). The activity encountered may be higher than that recognized in previous studies (2-4) due to species differences. In addition, the ovarian activity may not be detectable by antibodies directed against EGF of salivary origin. Since the antibody available for this study

failed to recognize porcine EGF from any tissue source, possible antigenic differences between salivary and gonadal EGF-like activity in the pig have not been resolved. However, in recent studies with cultured rat Sertoli cells there was also a marked discrepancy between receptor assay results and those by radioimmunoassay (5). It is now apparent that there are several growth factors which are recognized by EGF receptors (reviewed in Ref. 9). The distribution of these peptides and of related, potentially undiscovered ones, is presently uncertain. Such peptides are not generally recognized by antibodies to EGF.

While the current studies do not pinpoint the source of the ovarian EGF-like activity, the differences in concentration among the several classes of follicles implies local regulation of this activity. The apparent gradient between serum and small follicles further suggests possible ovarian production. In addition, the absence of detectable EGF-like in plasma may indicate that the EGF-like activity measured in serum derived from platelets or other cellular elements in blood which would not have access to follicular fluid. Such an interpretation would be consistent with the prior demonstration of a peptide resembling EGF or transforming growth factor-α in platelets (10,11). Finally, the distribution of EGF-like activity within the classes of follicles differs substantially from that encountered when similar samples were analyzed for insulin (no significant differences were observed) or for immunoreactive insulin-like growth factor-I (large follicles from cycling animals had the highest concentration) (12). Thus, the results seem unlikely to be accounted for by diffusion of peptides from serum for several reasons.

Irrespective of the source of ovarian EGF-like activity, important physiological effects could be exerted. At the concentrations measured in follicular fluid (>10 ng/ml) EGF has been shown to have potent mitogenic actions on granulosa cells of several species, including the pig (13). In addition, EGF exerts stimulatory activities on other growth-related parameters including ornithine decarboxylase (14) and thymidine incorporation into DNA (15) in pig granulosa cells. In contrast, the dominant effects of EGF on differentiated function of granulosa cells have generally been inhibitory. In particular, the peptide blocks the effects of follicle stimulating hormone and cyclic AMP on progesterone and estrogen biosynthesis (1,16). However, EGF has the potential to increase basal progesterone secretion under some circumstances (17). When these earlier results are integrated with the current observations regarding concentrations of EGF-like activity during follicular development, the evidence suggests that EGF-receptor-active peptides could function as local regulators within

within immature follicles to favor cell replication and to inhibit differentiation.

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