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CONTROL OF FIBRONECTIN SYNTHESIS BY RAT GRANULOSA CELLS IN CULTURE

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ABSTRACT: The secreted and cellular [^{35}S]methionine-radiolabeled proteins of cultured rat granulosa cells were separated by electrophoresis on sodium dodecylsulfate (SDS) polyacrylamide gradient slab gels. From 24 to 72 h of culture FSH increased the intensity of labeling of most of the secreted proteins. A 220,000-dalton protein, however, increased in intensity only in control cultures and became the major secreted protein after 72 h, comprising 20% of the total radiolabeled proteins. This protein was identified as fibronectin by immunoprecipitation. There was no increase in the secreted or cellular fibronectin in FSH- or testosterone- and insulin-treated cultures. These studies indicate that a component of extracellular matrix is a major secretory product of unstimulated immature granulosa cells. As hormones induce the differentiated functions of granulosa cells in culture, the secretion of fibronectin is inhibited.

In developing tissues, such as the follicle, cells interact in a number of ways in order to support morphogenesis and cytodifferentiation. Cell-cell interactions can exist between cells of the same type and between cell types of different embryonic origins. Communication can occur within a tissue by the secretion and transport of specific products to cells which have acquired the ability to respond to the transmitted signal. Proteins such as growth factors or components of extracellular matrix constitute one class of mediators of cellular communication which are important in the formation of a functional unit (1).

The granulosa cell is a hormonally responsive differentiating cell which plays an integral role in follicular development (2). The present study examines the radiolabeled proteins secreted by cultured granulosa cells from immature rats in the presence and absence of FSH, testosterone and insulin. The results indicate that hormones and the duration of culture have significant effects on the proteins secreted by granulosa cells.

MATERIALS AND METHODS

Immature Wistar CrL: (WI) BR rats were obtained from Charles River Canada (Montreal, Canada) and treated daily for 4 days with 1 mg diethylstilbestrol in 0.1 ml sesame oil by sc injection. Animals were killed at 25 days of age and the granulosa cells were recovered from the ovaries by puncturing the follicles with a fine needle, as described previously (3). Cell suspensions were plated in 24- multiwell Falcon tissue culture plates (Falcon Plastics, Los Angeles, CA) as 1.0-ml aliquots in modified Eagle's Minimum Essential Medium (MEM) and cultured at 37C in a humidified atmosphere of 5% CO_2 and 95% air to maintain the medium at pH 7.4(3). The cells were allowed to attach to the surface of the plate for 3 h before the addition of hormones (200 ng FSH-15/ml, insulin 5 ug/ml, $5x10^{-7}$M testosterone). After hormonal treatment the amount of DNA/well was approximately the same as controls and declined a maximum of 10% during the 72-h culture period. Fibroblasts were seen occasionally in the cultures but did not proliferate under the culture conditions employed. Thecal cells were not apparent morphologically, or biochemically as judged by undetectable levels of androgen.

After culturing the cells for various periods of time, the monolayer was washed with methionine-free MEM, and 0.5 ml of the same medium containing 10 μCi [^{35}S]methionine was added to each culture well. After 24 h of incubation, the medium was removed, phenylmethylsulfonyl fluoride was added, the suspension was centrifuged to bring down cell debris, and frozen. The cell monolayers were washed with 100 μl Dulbecco's phosphate-buffered saline which was discarded. SDS sample buffer was added to each culture well to remove the cells, which were then frozen.

Electrophoresis was performed on 5 to 15% polyacrylamide gradient slab gels with the use of the Laemmli SDS buffer system (4). All samples were reduced with β-mercaptoethanol prior to electrophoresis. Gels were fluorographed with the use of diphenyloxazole in acetic acid as previously described (5). Fluorograms were scanned on a Hoefer Scientific Instruments GS 300 scanning densitometer.

Fibronectin immunoprecipitation was carried out with a double-antibody precipitation procedure described previously (6). Rabbit anti-rat fibronectin (Calbiochem, CA) was the first antibody and goat anti-rabbit immunoglobulin (Sigma) the second antibody.

RESULTS

Electrophoretic profiles of the radiolabeled proteins secreted by granulosa cells are shown in Figure 1. During the first day of culture the cells secreted a large number of radiolabeled proteins into the medium and the hormones tested had little influence on the profiles. On the second day of culture there were differences in the intensities of secreted radiolabeled proteins between control and hormone-treated groups and between the pattern obtained in comparable cultures on the previous day. These differences were more apparent on the third day of culture. One of the major differences was the appearance of a 220,000-dalton protein in controls during the second and third days of culture, which remained low in hormone-treated cultures. A protein with mol wt of 200,000 increased during the period of culture only in FSH-treated cultures.

When radiolabeled proteins secreted by the control cultures from 48 h to 72 h were separated by electrophoresis under nonreducing conditions, the 220,000-dalton protein shifted to a molecular mass of approximately 400,000 (data not shown). Because fibronectin exhibited similar properties, control secreted proteins were immunoprecipitated with an antibody specific to fibronectin. The 220,000-dalton protein was found to be immunologically similar to fibronectin (Fig. 2,J). No detectable immunoprecipitate was found in medium of cultures treated with FSH or insulin and testosterone for 72 h (data not shown). Immunoprecipitation may not be stoichiometric, and the presence of other minor proteins in the 220,000 band cannot be excluded.

The relative amounts of fibronectin compared to the total radiolabeled proteins secreted were assess-

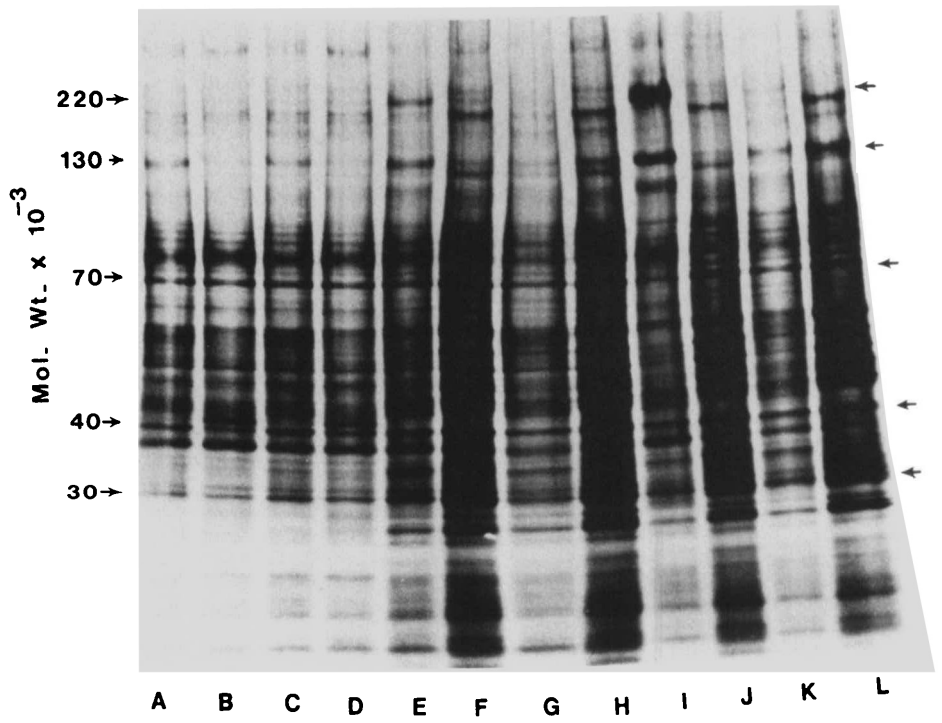

Fig. 1. Fluorogram of electrophoretic profiles of granulosa cell-secreted proteins. Radiolabeled secreted proteins were obtained from control (A,E,I); FSH-treated (B,F,J); insulin-and testosterone-treated (C,G,K); and FSH-, insulin-, and testosterone-treated cultures (D,H,L) at 24 h (A,B,C,D) 48 h (E,F,G,H) and 72 h (I,J,K,L).

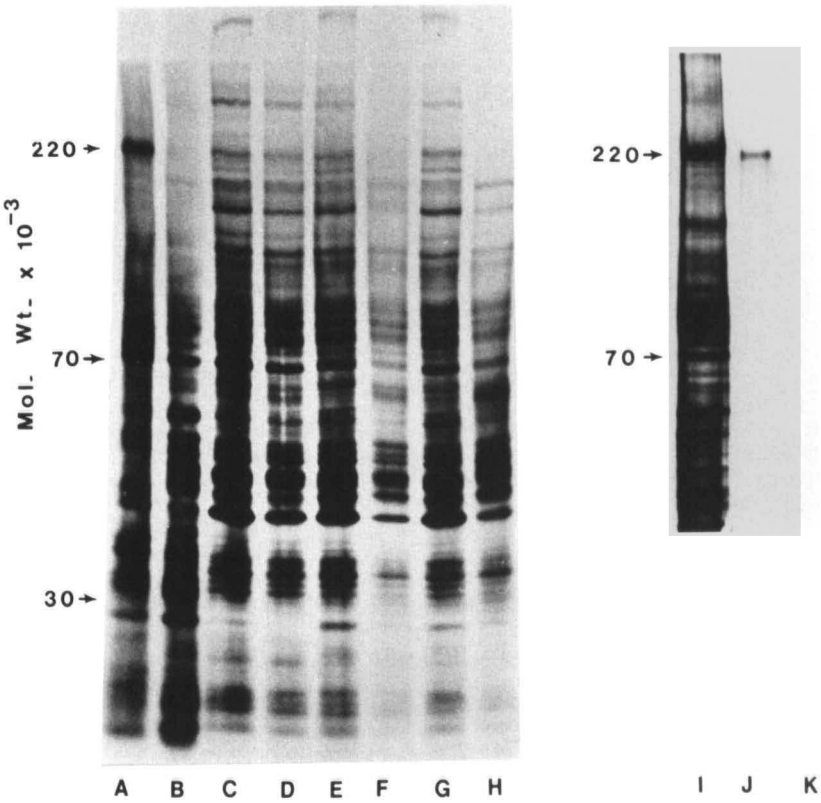

Fig. 2. Fluorogram of electrophoretic profiles of radiolabeled secreted and cellular proteins and fibronectin immunoprecipitates. Secreted proteins obtained from control (A) and FSH-, insulin-and testosterone-treated cultures (B) at 72 h. Cellular proteins from control (C,E,G) and FSH-, insulin-and testosterone-treated cultures (D,F,H) after 24 h (C,D), 48 h (E,F), and 72 h (G,H). Immunoprecipitates of secreted proteins obtained from control cultures after 72 h (I) with an antiserum specific to rat fibronectin (J) and nonimmune rabbit serum (K).

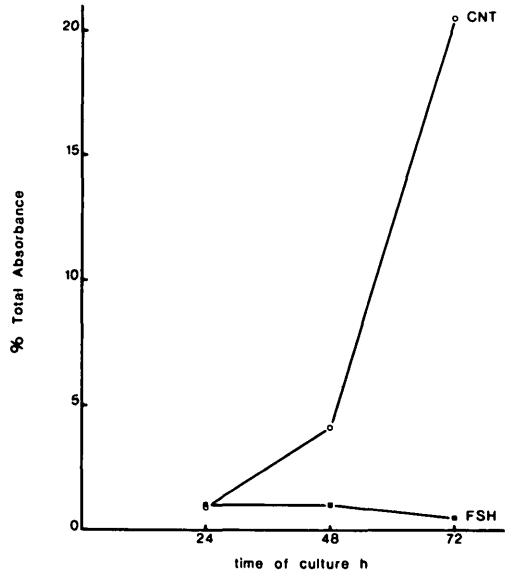

Fig. 3. The percentage of the total optical absorbance of the secreted radiolabeled proteins from control (o) and FSH (■)-treated cultures which is contributed by the 220,000-dalton protein (fibronectin). The fluorogram assessed is shown in Fig. 1.

ed on the fluorogram (Fig. 1) with a scanning densitometer. As shown in Figure 3, fibronectin secretion increased with time in control cultures to approximately 20% of the total optical absorbance of the radiolabeled proteins at 72 h. In FSH-treated cultures fibronectin comprised less than 1% of the total secreted proteins.

DISCUSSION

During follicular development, granulosa cells and thecal cells communicate to provide an environment in which the oocyte can develop normally. Since cells can interact by the production of soluble proteins, we have analyzed by electrophoresis on SDS polyacrylamide gradient slab gels the proteins secreted by granulosa cells as a function of time in culture and hormonal treatment.

The amount of a radiolabeled protein with mol wt of 220,000 secreted by control granulosa cells increased with time in culture and comprised 20% of the total radiolabeled protein by the third day. This protein had a mol wt and subunit structure similar to fibronectin. Its identity was established by immunoprecipitation with an antibody specific for fibronectin. The amount produced could not be due to the low level of contaminating fibroblasts. This is consistent with a previous observation that fibronectin is secreted by cultured bovine granulosa cells (7). Stimulation of the cultures with FSH or insulin and testosterone suppressed the increase in secretion of fibronectin, so that the amount comprised less than 1% of the total radiolabeled proteins on the third day of culture. An analysis of cellular radiolabeled proteins showed that fibronectin was a minor component, which did not change with time under control culture conditions and decreased slightly after hormonal stimulation. The hormonal inhibition of secretion of fibronectin is due therefore to a decrease in synthesis and not simply to an increase in deposition.

These studies indicate that a component of extracellular matrix is a major secretory product of unstimulated immature granulosa cells. Fibronectin plays an important role in cell adhesion, migration and cell shape. These functions are due to its ability to bind to collagen, heparin and the cell surface (8). Fibronectin also promotes the growth of several cell types by acting as a "competence factor" (9). The observations that fibronectin was produced in reduced amounts in transformed cells suggested originally that the synthesis of this protein may be related to the state of differentiation of the cell. This notion has been supported by the inhibitory effect of fibronectin on glycosaminoglycan synthesis by chondrocytes and on myoblast fusion (8). More recently, fibronectin has been shown to regulate adipocyte differentiation by inhibiting normal gene expression of lipogenic proteins, possibly by interfering with cytoskeleton changes necessary for normal gene expression (10). The diverse functions of fibronectin imply that it may play an integral role in normal cell functions and cell-cell communication.

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