EXPERIMENTAL BIOLOGY

Ultrastructural Visualization of Protein-4 Binding Insulin-Like Growth Factors in Cow Ovarian Follicles

A. L. Glazyrin, D. G. Armstrong*, K. G. Guttieras*, T. A. Bramley*, R. Webb*, and S. I. Kolesnikov

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Protein-4 that binds the insulin-like growth factors (IGFBP-4) was visualized in the basal membrane of some vessels and follicles and in extracellular membrane surrounding thecal cells in middle-sized cow ovarian follicles. Examinations of serial sections of thecal granular cells showed highly specialized sites for detection of this protein on plasma membranes and highly specialized endosomes responsible for accumulation of IGFBP-4 in granular cells. These data point to an active role played by the extracellular ovarian matrix in the binding and accumulation of insulin-like growth factor and to high specificity of internalization and exocytosis of IGFBP-4 by ovarian granular cells.

Key Words: *insulin-like growth factor; ovary; electron microscopy; immunocytochemistry*

The development of mammalian oocytes is regulated by hormones and some local factors that mediate and modify the effects of these hormones [1,3]. The understanding of the mechanisms responsible for the physiological effects of these hormone-like factors is essential for the regulation of human and animal reproductive function. The family of insulin-like growth factors (IGF) includes IGF-1 and IGF-2, specific receptors for IGF-1 and IGF-2, six types of binding proteins (IGFBP 1-6), and specific proteases for each type of binding proteins. Complex interactions between these proteins have been little studied, and the results of *in vitro* experiments should be interpreted with care because of the intricate interactions between the components of this system *in vivo* [8].

We investigated the ultrastructural localization of IGFBP-4 in cow ovaries. This protein specifically binds IGF and regulates their biological activity; a specific protease for it has been detected [5]. IGFBP-4 was detected in many tissues, including granulocytes of the rat atretic follicles, human placenta, and prostatic carcinoma [7]. In humans, the content of IGFBP-4 is higher in follicular fluid from the follicles with a high androgen/estrogen ratio, indicating an inhibitory role of this protein [3]. IGFBP-4 inhibits the mitogenic effect of IGF on vascular smooth-muscle cells [2]. The localization of this protein in the ovaries has not been studied by ultrastructural methods.

MATERIALS AND METHODS

The ovaries from 6 cows were obtained from a local slaughter house in Scotland. Follicles (5-8 mm in size) were dissected immediately and fixed in 4% paraformaldehyde. They were then embedded in LRWhile plastic, ultrathin sections were mounted onto nickel grids, and incubated for 4 h at 18-20° in 5% bovine serum albumin in Tris buffer (pH 7.4) with 1% Tween-20, after which the grids were placed in a drop of

Institute of Pediatrics and Human Reproduction, East Siberian Research Center, Siberian Division of Russian Academy of Medical Sciences, Irkutsk; *Department of Development and Reproduction, Roslin Institute, Midlotian, Scotland

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primary antibodies in Tris buffer and left overnight at 4°C. Then the grids were thoroughly washed in Tris buffer and put into a drop of protein A-colloid gold, dissolved in the same buffer (1:200). After a 45-min incubation, the grids were washed and contrasted with uranyl acetate. In the control the grids were incubated only in the protein A-colloid gold solution, and primary antibodies were replaced by nonimmune rabbit serum. Only several scattered particles were seen on the control sections (Fig. 1).

RESULTS

Immunocytochemical analysis showed specific labeling of the extracellular matrix, primarily of collagen fiber cords round thecal cells (Fig. 2). Secretory granules inside individual thecal cells (Fig. 3) and follicular and vascular basal membranes were labeled. The labeling of vascular basal membranes was mosaic: some of them were labeled slightly or had no specific label at all, while the neighboring vessels could be well impregnated with specific label. The basic matter of ovarian connective tissue was labeled negligibly, in contrast to individual cords of collagen fibers, which were strongly labeled. Fibroblasts were poorly labeled. In follicular granulocytes, some sites of plasma membranes and vacuoles inside the cells were stained (Fig. 4). On serial sections, the label was focused in serially repeated sites of the granulocyte plasma membrane in the same endosome-like structures inside granular cells (Fig. 5). The majority of endosomes and the major part of granulocyte plasma membrane had no specific label.

This study demonstrates the binding of IGFBP-4 to the extracellular matrix in cow ovaries. Previously it was shown that many protein growth factors bind to proteoglycans and/or glycosaminoglycans, and these reactions modify the biological activity of these factors [9]. The components of extracellular matrix and IGFBP are related. IGFBP belongs to a small group of proteins possessing a specific binding site for glycosaminoglycans; high affinity of some IGFBP for glycosaminoglycans has been demonstrated *in vitro* [1,6]. Since IGF are located in the extracellular matrix and therefore, similar location of binding proteins can be expected [4]. However, studies of human IGFBP showed

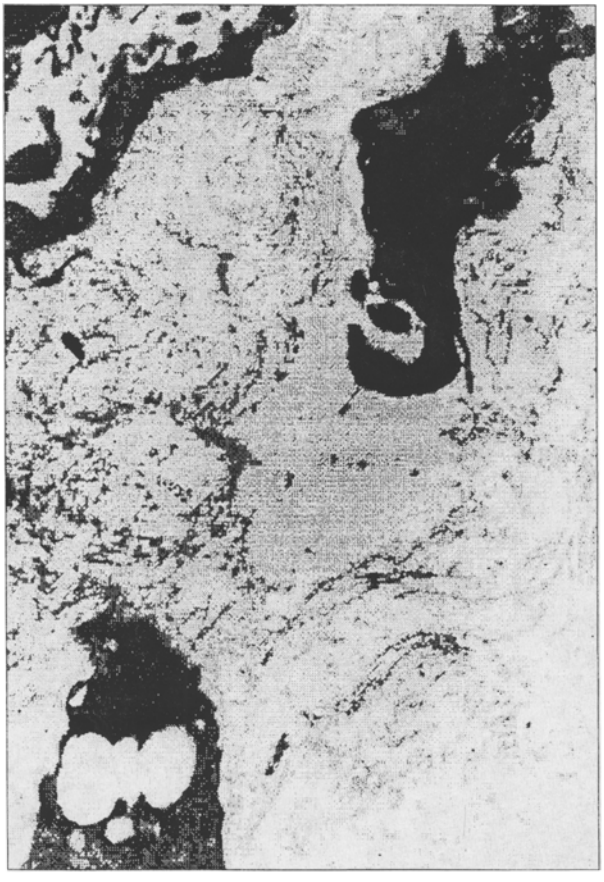

Fig. 1. Stroma of cow's ovaries. Control section, primary antibodies replaced by nonimmune rabbit serum. Chaotically scattered gold particles. x5000.

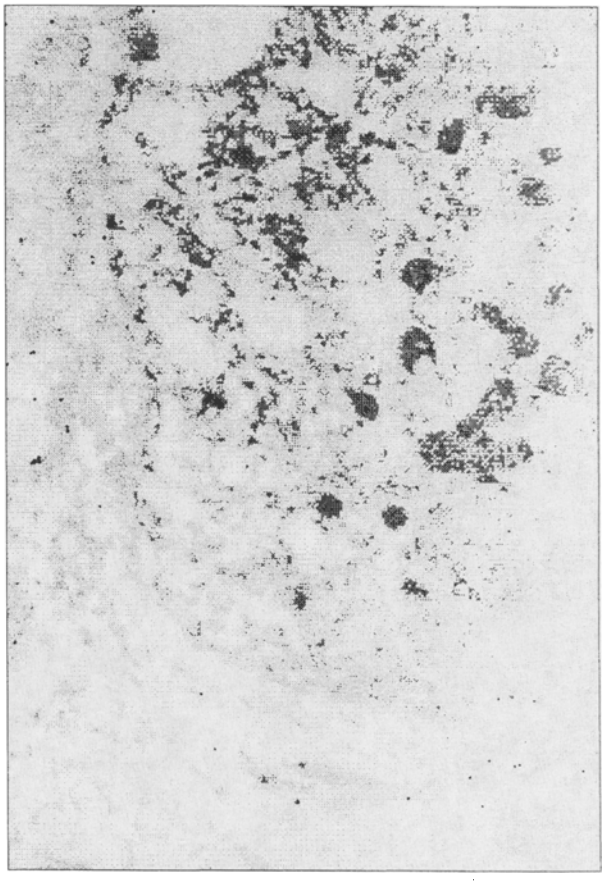

Fig. 2. Thecal cell in cow ovarian follicle. Cell cytoplasm is not labeled but the adjacent sites of the extracellular matrix contain IGFBP-4. x7000.

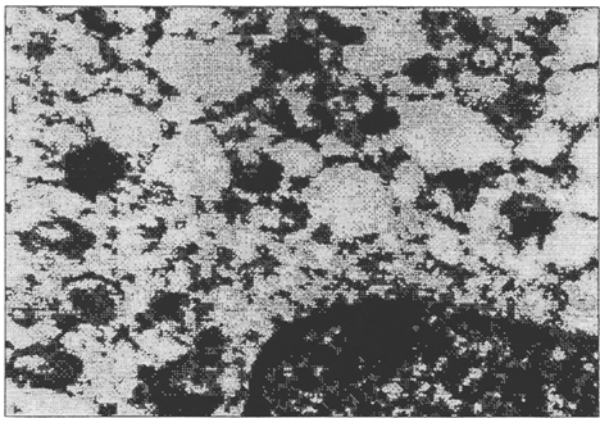

Fig. 3. Specific dark granules in the perinuclear area of thecal cell contain considerable amounts of IGFBP-4. ×7500.

that human IGFBP-4 does not possess specific binding sites for glycosaminoglycans [7].

In this study we revealed numerous IGFBP-4 bound to basal membranes and extracellular matrix round thecal cells, i.e., in sites rich in proteoglycans. This may be due to the species specificity, binding through other biological determinants, and the fact that primary antibodies were 50% cross-reactive with IGFBP-2 (as specified by the manufacturer). Although the latter is important for the interpretation of our results, study of granular cells using anti-IGFBP-2 antibodies from the same company revealed an essential difference in their labeling at the optic and electron microscopy levels and in immunoblotting. Thus, in general the label visualized IGFBP-4 but not other similar proteins.

The function and origin of IGFBP-4 in the extracellular matrix are not clear. This factor is delivered via the bloodstream [1] and Cs produced in the ovaries [3]. Its function in the extracellular matrix is unclear. On the one hand, IGFBP-4 is a potent inhibitor of IGF, and binding of this growth factor to sites of its transport through basal membranes and extracellular matrix may markedly decrease its accessibility to target cells. On the other hand, the presence of binding proteins at this site suggests that extracellular matrix serves as a reservoir of growth factors from which they can be released when required. Specific proteases for IGFBP are probably involved in this release [3].

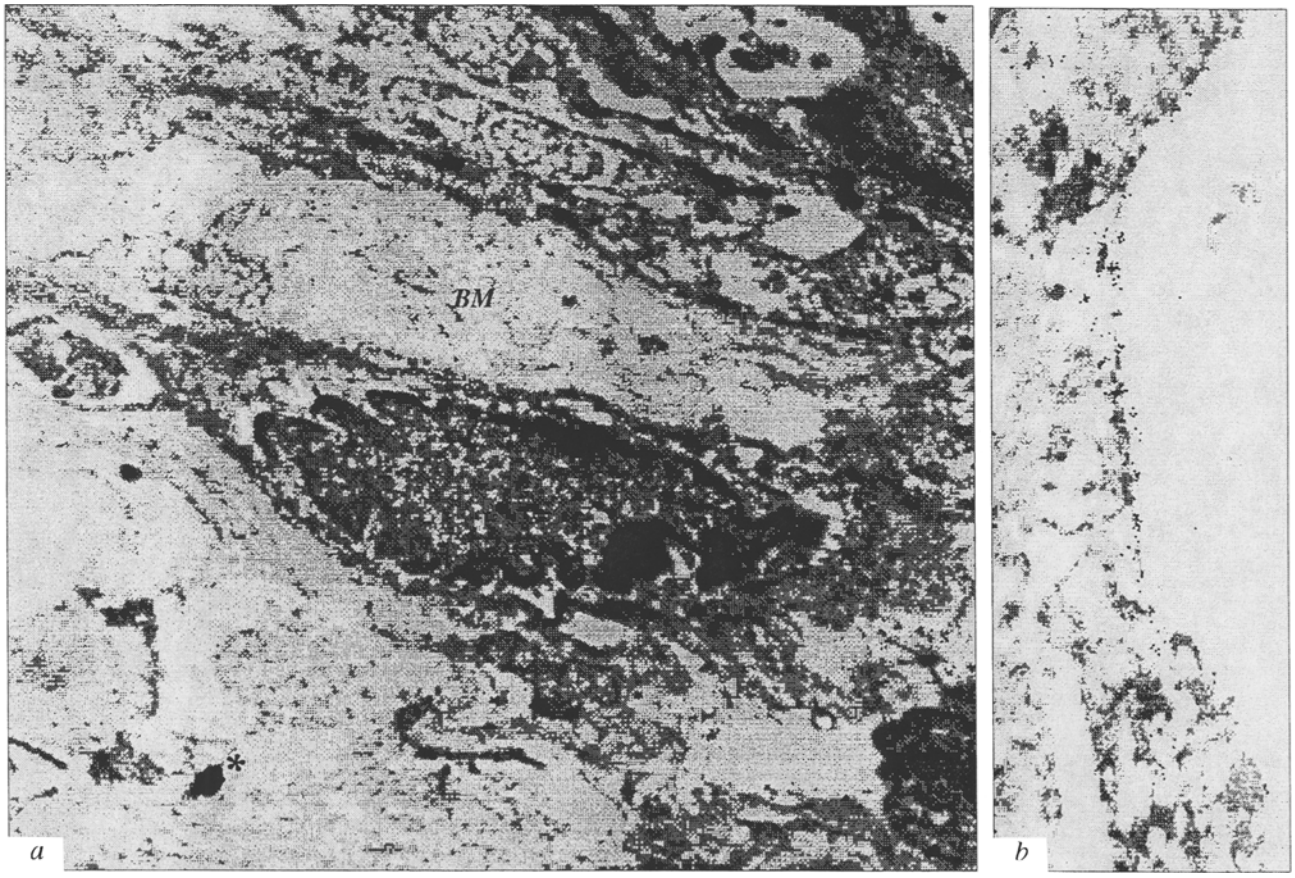

Fig. 4. IGFBP-4 in the internal ovarian theca (*a*) and in follicular granular cell (*b*). *a*) intense labeling of the vascular basal membrane (BM); deeper layers of connective tissue and endotheliocytes are poorly labeled (*). ×7500. *b*) intensively labeled plasma membrane and perinuclear endosome. ×7000.

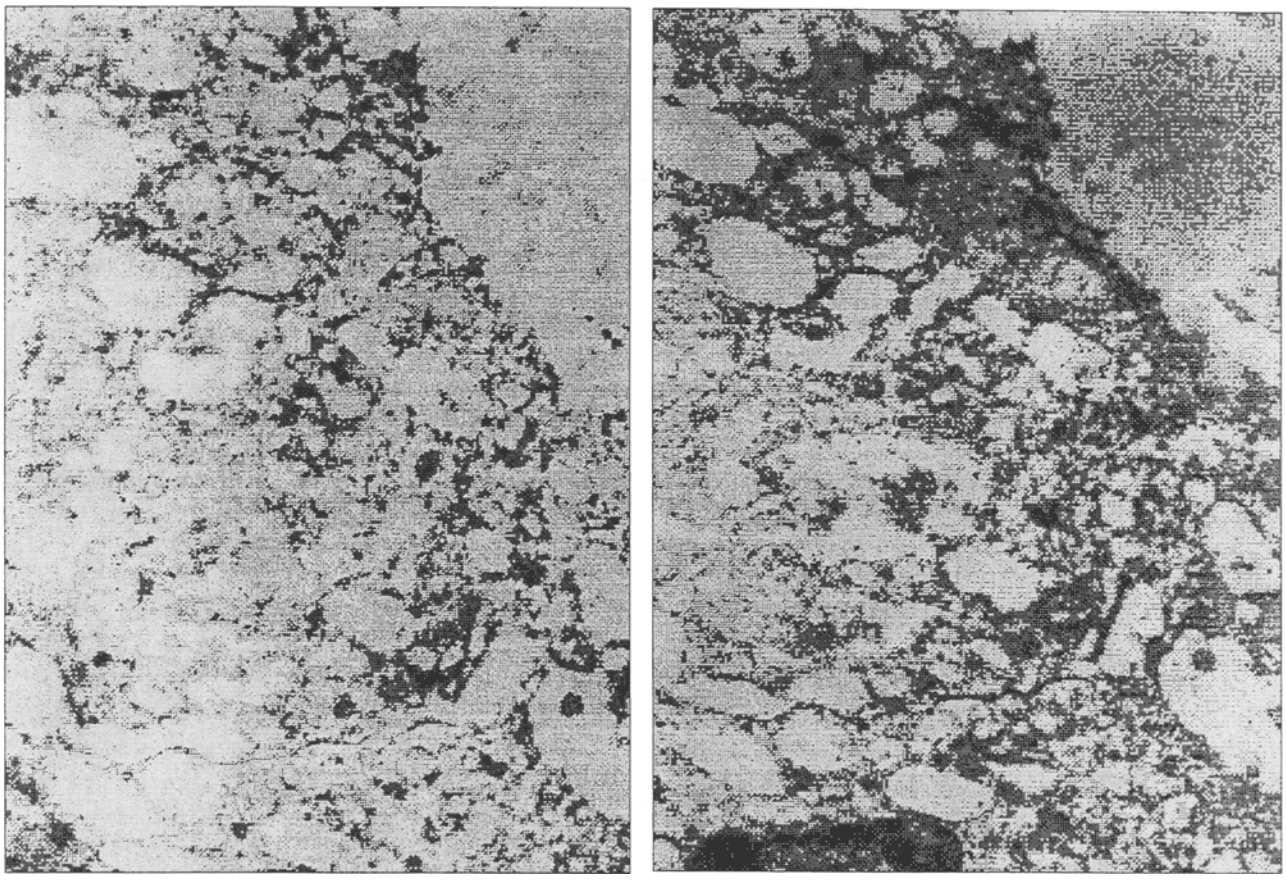

Fig. 5. Serial sections of cow ovarian granulocyte. The same sites (endosomes) of plasma membrane are labeled. The majority of endosomes are not labeled. ×7000.

In this study we demonstrated the presence of specific strictly determined binding sites for IGFBP-4 on the granulocyte membrane and highly specialized endosomes for the accumulation of this protein. Intense labeling of these sites was observed on electron microscopic preparation (Fig. 5). In the majority of cases the sites of plasma membrane and granulocyte endosomes displayed no specific labeling for IGFBP-4. This proves high specificity of binding, internalization and/or production, and release of this protein. The physiological significance of these highly specialized endosomes is not clear.

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