Journal of Applied Pharmaceutical Science Vol. 4 (06), pp. 001-008, June, 2014
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2014.40601
ISSN 2231-3354

Identification, Isolation and Characterization of IGF Binding Proteins as a Secretable Proteins of Human Endometrial Stromal Cell Culture

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ARTICLE INFO

Article history:
Received on: 03/02/2014
Revised on: 27/02/2014
Accepted on: 26/04/2014
Available online: 28/06/2014

Key words:
IGFBPs; Cell culture;
Endometrial Stromal cells.

ABSTRACT

IGF-1, a high potent mitogenic factor with a dual function is believed to participate with female steroids and other growth factors to prepare endometrium receptivity for successful embryo implantation and further development. Stromal cells forms the functional receptive layer of endometrium structure. Analysis of the conditioned/defined medium of pure cultured human endometrial stromal cells have revealed that stromal cells in a monolayer culture secrete and produce three different types of proteins in a varying amounts. These proteins exhibit a high specificity as well as a high binding affinity for the radiolabeled IGF-1 peptide. The molecular weights of these proteins have been determined by SDS-Page electrophoresis after cross-linking with the radiolegand IGF-1 and then detected with autoradiography. By comparison to the migration of high molecular weights protein markers, these proteins have been identified to correspond to the IGFBP-1 (31 KDa), IGFBP-2 (36 KDa) and IGFBP-3 (45 KDa). The secretion of these binding proteins (IGFBP-1, -2, -3) by endometrial stromal cells may support the view of their biological importance in controlling the delivery and bioavailability of the high mitogen IGF-1 peptide to their nominative type-1 IGF-receptor on cell surface, thereby modulating its action. It seems likely that these IGFBPs may play a key role in switching on/off IGF-1 peptide action , thereby avoiding the uncontrolled proliferation effect of the IGF-1 that favor endometrium cancer development.

INTRODUCTION

Insulin-like growth factors IGF-I and IGF-II are a family of polypeptide chains with a high sequence similarity to each other as well as to insulin (Zapf *et al.*, 1981). IGF-I contains 70 amino acids a single polypeptide chains with three disulphide bridges and has a molecular weight of about 7.649 KDa (Rinderknecht, *et al.,* 1978). IGF-I and II are potent mitogenic peptides elicit the same biological response as insulin (Rechler, *et al.*, 1985). The primary purpose of IGF-I is to stimulate cell growth, every cells in the human body can be affected by IGF-I, but cells in muscle, cartilage, bones, liver, kidney, skin tissues and nerves tend to be the most positively affected cells (Rabinovski, 2004)). There is also increasing evidences, that these growth factors may act at multiple levels in regeneration response. IGF-I is known to promote nerve elongation and branching, it may also promote satellite cells proliferation and differentiation and muscle hypertrophy (Rabinovsky, 2004). At the angiogenesis level, IGF-I is also known to promote the angiogenetic process in regenerating skeletal muscle by activating VEGF and VEGF-receptor, It may also promote the activation of muscle stem cells during regenerative process (Rabinovsky, 2004). IGF-I peptide is mainly produced by the liver in response to growth hormone from pituitary galnd as an endocrine hormone (Rosenfeld *et al.,* 1986) as well as locally by target tissues in a paracrine/autocrine fashion (Jones *et al.,* 1995). IGFs peptides interact with specific cell surface receptors designated as type I and type II IGF-receptors, and can also interact with insulin receptor. The mitogenic effects of the IGFs peptides are mediated mainly through the interaction with type I IGF-receptor, which like insulin receptor in having tyrosine kinase activity (Gross *et al.,*2004). In biological fluids, IGF-I and II are normally bound to a specific proteins known as binding proteins IGFBPs, that have a higher affinity for IGFs peptides than do the type I IGF-receptors on cell surface. Therefore, these IGFBPs act not only as a carrier of IGFs peptides, thereby extending the half-life of these IGFs but also functions as a modulators of IGFs availability and activity (Jones *et al.*, 1995).

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This availability of IGF-1 to its receptors is also further regulated by certain IGFBP-specific proteases that are produced by multiple body tissues and are also found in the circulation (Zapf *et al.,* 1995). Six IGFBPs , IGFBP-1 through IGFBP-6 have been described (Rechler, 1993).

IGFBP-1 is also known as a Placental protein PP-12. It is 31 KDa protein that contains the amino acid sequence RGD as a recognition site for the membrane integrin receptor. IGfBP-1 is produced by liver, decidua and kidney. Its serum levels is controlled by insulin, corticosteroids, as well as by nutritional status. During fasting, the IGFBP-1 serum level is increased and this increase curbs the hypoglycemic effect by binding to IGF-1 thereby, decreasing the availability of IGF-1 peptide to its surface receptor (Collet-Solberg and Cohen, 1996). It has also been found that the movement of free IGF-1 across the endothelial membrane may be partly controlled by IGFBP-1 and appears to be insulin-dependent in certain tissues (Lewitt *et al.*, 1993).

IGFBP-2 is a 36 KDa protein that also contains the amino acid sequence RGD as a recognition site for the membrane integrin receptor. IGFBP-2 appears to possess multiple biological functions beyond the modulation of IGFs peptides activity through sequesteration of these peptides and inhibiting cell growth as shown by the addition of IGFBP-2 to a culture media of multiple cell lines (Jones and Clemmons, 1995). It is also involved in regulation of both cell adhesion and cell migration (Schutt *et al.,* 2004). Age is the major regulator of IGFBP-2 levels in serum , a high level has been observed in early and late life , while low one is noticed in young adults. The serum IGFBP-2 levels are also elevated in response to somatotropin deficiency or in fasting situation particularly in a case of protein restricted diet (Collet-Solberg and Cohen, 1996). It has also been reported that IGFBP-2 is often high expressed in malignant tumor cells, but decreases upon remission (Muller *et al.,* 1994). This elevated level expression is reflected by increased serum concentration of IGFBP-2 in tumor patients, which is often correlated with tumor malignancy. This makes IGFBP-2 a potential progression-dependent tumor biomarker and suggested a functional role for IGFBP-2 in tumor progression. (Baron-Hay *et al.,* 2004).

IGFBP-3 is a 264 amino acids polypeptide chain with a molecular weight of about 29 KDa. in a free nonglycosylated form, but in serum IGFBP-3 is found in glycosylated form with a molecular weight of 40-45 KDa. glycoprotein complex consisting of IGFBP-3 itself and IGFs molecules in a binary complex (Daughaday *et al.* 1989; Sigh, 2000), sometimes IGFBP-3 contains also ALS, a leucine rich glycoprotein beside IGFs molecules in a heterotrimeric complex with a molecular weight of a 150 KDa (Baxter *et al.,* 1989). Approximately, 95 % of IGF-I and IGF-II are bound to IGFBP-3, which makes this protein as the major carrier of insulin-like growth factors in the circulation. IGFBP-3 is secreted by the liver in response to growth hormone from pituitary gland, it is a growth hormone-dependent (De Mellow *et al.,* 1988) therefore, it is useful in evaluation of human growth hormone secretion and disorders. IGFBP-3 serum levels are under complex regulation by age, somatotropin and pathological status. Its concentration increases in childhood to a peak around puberty then decline by adulthood. Somatotropin increases IGFBP-3 mRNA levels in multiple tissues suggesting that somatotropin might have a direct role in the regulation of IGFBP-3 synthesis. In some pathophysiological conditions, It was found that IGFBP-3 was significantly increase in medulloblastoma patients than control (De Bont *et al.,* 2008). In the context of predicting cancer risk, there has been considerable recent reports that a high serum IGFBP-3 level reduces the relative risk of developing breast cancer predicted by high IGF-1 level in premenopausal women (Hankinson *et al.,* 1998). High serum IGFBP-3 also tends to attenuate prostate and lung cancer risk in patients with high IGF-1 peptide (Chan *et al.,*1998; Yu *et al*., 1999).

IGFBP-4 is the smallest of the six high affinity IGFBPs and is the second abundant circulating one. IGFBP-4 is 237 amino acid polypeptide chain that contains N-linked gylcosylation site and commonly exists in biological fluids as a doublet 24 KDa. non-glycosylated form and 28 KDa. glycosylated form (Wetterau *et al.,* 1999). It has been identified in biological fluids including serum, follicular fluid, interstitial fluid, and synovial fluid (Rajarm *et al.,* 1977). In rat serum, it is the most abundant IGFBPs after IGFBP-3. Northern blot analysis has revealed that IGFBP-4 mRNA is widely expressed in adult rat tissues as adrenal gland, testis, spleen, lung, kidney, and the liver being the site of higher expression (Shimonaka *et al.,* 1989). It is also expressed in rat small intestine (Shoubridge, *et al.,* 2000), smooth muscle (Smith *et al.,* 2001), skeletal muscle (jennische and Hall, 2000), Pancrease (Hill *et al.,* 1999), uterus and placenta (Cirro and Pintar, 1997), overian tissue (Zhou and Bondy, 1993), human prostate (Thomas *et al.,* 2000), and bone tissues (Mohan *et al.,* 1995a). This wide expression of IGFBP-4 in various multiple tissues suggested its developmental regulation by hormones, cytokines and other agents in a tissue specific manner. In vitro studies, it has been shown IGFBP-4 inhibits IGF-I activity by a competitive binding to free IGF-I, thereby decreasing the bioavailability of free IGF-I in extracellular environment and IGF-I cell surface receptor (Mohan *et al.,*1995).

IGFBP-5 is a 252 amino acid polypeptide chain with 29 KDa molecular weight in free form and 32 KDa molecular weight in glycosylated form. It is expressed with a hydrophobic signal sequence of about 19 – 20 amino acid residues that is characteristic of secretable proteins (Beattie *et al.,* 2006). IGFbP-5 is a highly basic protein with an isoelectric point of about 8.5. One of the most significant features of its primary sequence is the presence of a large number of highly conserved cysteine residues. There are 18 cysteine residues in IGFBP-5, 12 in N-terminal region and 6 in C-terminal region (Shimasaki *et al.,* 1991). It has also been assigned a disulphide bond linkage in N-terminal region/domain as well as in C-terminal domain. These data indicate that IGFBP-5 disulphide bonded within N-terminal domain and C-terminal domain, this pattern of intra-domain disulphide bonding has led to a consenus model of IGFBP-5 structure as comprising of discrete N-terminal domain and C-terminal domain joind together by a bridge region (Kalus *et al.,*

1998). This central hinge region was found to be the site of the major post-translation modification of this protein. IGFBP-5 is found in a high amount in bone cells, cerebrospinal fluids, connective tissues and kidney is the major organ of its production (Mohan *et al.,* 1995).Serum level of IGFBP-5 declines after puberty. However, its expression was observed to be increased in involuting prostate (Thomas *et al.,* 1998). A similar increase of IGFBP-5 expression has also been occurred in involuting of mammary gland at the end of lactation (Beattie *et al.,* 2006) The over expression of IGFBP-5 has shown to inhibits MCF7 breast cancer cell tumors in vivo and in vitro and is associated with increased survival of breast cancer patients (Ahn *et al.,* 2010).

IGFBP-6 is a member of IGFBPs family and has a molecular weight of 32 KDa and contains O-glycosylation site. It exhibits a significant binding preferences for IGF-II rather than IGF-I peptide. It is expressed by multiple cells including ovarian cells, prostate cells, fibroblast cells (Bach *et al.,*1994), and over expressed in HEK-293 cell line (Losef *et al.,* 2008), IGFBP-6 is in fact a growth inhibitory protein that not only regulates the bioavailability of IGFs peptides to cell surface receptors, but also exerts an intracellular action via translocation to the nucleus. KU80, Ku70, histone H2B and importin-alpha are nuclear proteins involved in DNA stability and repair process. Recently it has shown that IGFBP-6 is co-purified with the above mentioned proteins by tandem-affinity technique. This art of co-purification indicates a selective and specific interact of the IGFBP-6 with Ku80, where the IGFBP-6 chain has two binding sites for Ku80 in both N-terminal domain and C-terminal domain and one of these binding sites can also bind importin-alpha which may selectively compete with Ku80, thereby regulating its trafficking to the nucleus (Losef *et al.,* 2010). The over expression of IGFBP-6 in HEK cells alter the cell survival by potentially regulating the availability of Ku80 required for DNA repair process and stability (Losef *et al.,* 2010).

These six high-affinity IGFBP-1 to IGFBP-6 are secretable proteins, synthesized and secreted by different organs and multiple target tissues in a paracrine/autocrine fashion and are in fact considered as an effective member of the IGF-system/or axis. This system or axis includes the mitogenic peptides IGF-1 /IGF-II, IGF-receptors IGFBPs and IGFBPs-proteases. These IGFBPs work in this sytem not only as a carrier of the mitogenic IGF peptides , thereby increasing their half-life, but also modulate the IGFs peptides function in both dependent and independent manner. Endometrium, the mucous lining layer of uterine cavity is an epithelio-mesenchymal structure whose biological role is to provide an optimum environment for the implantation and development of fertilized ovum. To fulfill this function, the endometrium must undergo a complex series of changes involving proliferation, differentiation of both epithelial and stromal cells during regulatory cycle (Bell, 1983). These cyclic changes are primarily controlled by estradiol, progesterone and recently it became apparent that growth factors , their receptor and their binding proteins are likely to play an important role in regulating female reproductive tract physiology. Several reports show the presence of growth factors and their receptors in reproductive tissues of a variety of species (Ghahary *et al.,* 1989). Immunohistochemical studies has demonstrated the localization of mRNA for IGFBP-1 to IGFBP-4 in human endometrial epithelial and stromal cells during late leutal phase of menstrual cycle and also in human decidual cells during early pregnancy (Xin-Min *et al.,* 1994) and in baboon decidual cells throughout pregnancy (Pazleabas, 1989).

This expression of mRNA for both IGFs peptides and IGFBPs proteins in uterine tissues shed some light on the probable molecular interplay of these IGFBPs and steroid hormones in optimizing the endometrial tissue for successful implantation of fertilized ovum and subsequent development of the embryo. In this study we introduce evidences that endometrial stromal cells produce and secret some individual of IGFBPs we have identified three of these IGFBPs proteins and studied their binding activities with the radiolabeled 125-IGF and characterized their molecular weight by autoradiography.

MATERIAL AND METHODS

Endometrial stromal cell culture

Endometrial samples were obtained from hysterectomy specimens in the proliferative phase of menstrual cycle after patients informed consent. Stromal cells were isolated from epithelial cells as described previously (Satyaswaroop *et al.,* 1979), with a slight modification. Briefly, endometrial samples were collected in ice cold Minimal Essential Medium (MEM) with L-glutamine containing 10 ug/ml imipenem, 200 U/ml nystatin and immediately transported to the laboratory. The samples were minced with scissors, washed twice with 20 ml phosphate buffer saline, pH 7.4 under sterile conditions then centrifuged at 1000 rpm for 5 min. at room temperature. The pellet was resuspended in 20 ml sterile medium containing 100 mg collagenase type-I for enzymatic digestion. Digestion was carried out in 75 ml culture flask at 37 C for 2 – 3 hours under continuous shaking. After digestion the sample was centrifuged at 1000 rpm for 5 min. at room temperature, the supernatant was discarded, and the pellet was resuspended in 5 ml fresh medium and filtered through 50 um nylon sieve to remove glandular epithelial cells. The filtered cell suspension then seeded in 75 ml culture flask containing 10 ml culture (MEM) with L-glutamine and supplemented with 10% fetal calf serum, 10 ug/ml imipenem, and 50000 U/ml nystatin. Cells were grown to confluence within 7 days, then they were further subcultured and passaged until a monolayer of the stromal origin was obtained. Culture prepared by this method contain pure stromal cells as verified by negative staining to cytokeratin, desmin as well as mascular actin antibodies and positive staining with vimentin (Irwin *et al.,* 1989). Cells were maintained in culture for more than 15 passages.

Proliferation of stromal cells

The mitogenic activity of EGF peptide on the endometrial stromal cells has been studied by plating 30000 cells in 24-well plate (15 mm in diameter). Cells were allowed to grow

for six days in 2 ml culture medium (MEM, with L-glutamine supplemented with 10% steroid-free FCS) containing 1, 10, 100 nM EGF. The culture media were renewed on alternate days. After six days of incubation, the culture media were removed and the monolayer washed twice with fresh medium and then detached by a short incubation in 1 ml Puck solution (0,05% trypsin, 0,02% EDTA) at 37 C for 15 min. and then counted in coulter and finally expressed as percentage of the control values.

Preparation and harvesting of conditioned media

Stromal cells were seeded at 100000 cells in 75 ml culture flask containing 10 ml culture medium (MEM) with L-glutamine and supplemented with 10% steroid- free FCS calf serum and 10 nM EGF, 10ug/ml imipenem, and 50000 U/ml nystatin. The cells were allowed to grow for six days till confluence the medium was changed on alternate days. By confluence the steroid-free calf serum medium was removed and the cells in monolayer was washed twice with 30 ml with FCS-free culture medium (MEM) with L-glutamine under sterile condition then allowed to grow in 10 m FCS- free medium for four days, this conditioned /defined medium was changed on alternate days, then pooled. The pooled conditioned/defined medium was then dialysed using a cellophane bag for 2 days against distelled water at 4 C. The dialyzed medium was shortly centrifuged for 5 at 12000 rpm and 4 C. the supernatant was pooled and lyophylised over night in 100 ml lyophylisation flask. The powdered obtained was then reconstituted in 10 ml phosphate buffer pH 7.4 and used for further studies and determinations.

Determination of conditioned medium protein content

Protein content of conditioned medium was determined according to the method (Bradford, MM. 1976). 10 mg % bovine serum albumin was used as a protein standard solution. Three set of test tubes assigned as blank, standard and test were used, black contains 100ul Phosphate buffer, standard contains 100 ul bovine serum albumin and test contains 100 ul reconstituted conditioned medium, to each of these tubes 1 ml color reagent (10% w/v Coomassie Brilliant Blue G-250, 4.7% w/v ethanol and 8.5% w/v phosphoric acid) was added, stand for 5 min. at room temperature and the absorbance was measured at 595 nm against reagent blank. The values of protein content in conditioned medium was calculated according to Bear Lambert law for colorimetric analysis.

Binding studies

Binding of condition medium proteins with radiolebeled IGF-1

Binding assay of conditioned medium proteins with a radiolabeled IGF-I was carried out according to the method (Corleta *et al.,* 1992), with a little modification. Briefly 100 ul aliquots of reconstituted condition medium proteins with serial concentrations (15 - 75 ug protein) were incubated with 50 ul lebeled IGF-I (containing 50000 cpm) in absence and presence of 500 ng native IGF for 2-3 hours at room temperature in a final volume of 500 ul binding buffer (20 mM Hepes, pH 7.4, 0.1 % BSA). The bound IGF-I with conditioned medium proteins was determined by the addition of 500 ul (1% activated cooled charcoal+ 0.01% dextrane mixture in a binding buffer) then incubated in ice bath for 15 min. Incubation was terminated by centrifugation at 12000 rpm for 15 min. at 4 C. 500 ul of the supernatant containing the bounded radiolabeled IGF-I with conditioned medium protein was counted in gamma sprctrometer. The free unbounded radiolabeled IGF-I was adsorbed by charcoal and removed by pellet fraction. The non-specific binding was determined in the presence of 500 ng native IGF-I.

Saturation of conditioned medium proteins with labeled IGF-I

The saturation of conditioned medium proteins with radiolabeled IGF-I was determined similar to the binding study described previously. Briefly, 10 ul of reconstituted conditioned medium containing 30 ug proteins were incubated with serial concentrations of radiolabeled IGF-I (20000-200000 cpm) in the absence and presence of 500 ng native IGF-I for 2 – 3 hours at room temperature in a final volume of 500 ul binding buffer (20 mM Hepes, pH 7.4, 0,1 % BSA). The bound IGF-I with conditioned medium proteins was determined by the addition of 500 ul (1% activated cooled charcoal+ 0.01% dextrane mixture in a binding buffer) then incubated in ice bath for 15 min. Incubation was terminated by centrifugation at 12000 rpm for 15 min. at 4 C. 500 ul of the supernatant containing the bounded radiolabeled IGF-I with conditioned medium proteins was counted in gamma sprctrometer. The free unbounded radiolabeled IGF-I was adsorbed by charcoal and removed by pellet fraction. The non-specific binding was determined in the presence of 500 ng native IGF-I.

Affinity cross-linking and autoradiographgy

To determine the approximate molecular mass of conditioned medium proteins, firstly, a binding study was carried out with the radioliganed IGF-I then followed by cross-linking and covalently binding of the radioligand with the conditioned medium proteins and then electrophoresis and autoradiography. Briefly, an aliquot 100 ul of reconstituted conditioned medium containing 100 ug proteins were incubated with radiolabeled IGF-I (250000 cpm) for 2 – 3 hours at room temperature in a final volume of 500 ul binding buffer (20 mM Hepes, pH 7.4, 0.1 % BSA). The bound IGF-I with conditioned medium proteins was determined by the addition of 500 ul (1% activated cooled charcoal+ 0.01% dextrane mixture in a binding buffer) then incubated in ice bath for 15 min. Incubation was terminated by centrifugation at 12000 rpm for 15 min. at 4 C. For covalent binding and cross-linking reaction of radioligand IGF-I with conditioned medium proteins. 500 ul aliquot of the supernatant containing the bounded radiolabeled IGF-I with conditioned medium proteins was mixed well with 30 ul (1 mM) disuccinimidyl suberate freshly prepared in dimethylsulphoxide (DMS) and incubated in ice bath for 15 min. The cross-linking reaction was terminated by the addition of 70 ul stop buffer (500 mM trishydrochloride pH 7.4, containg 100 mM EDTA). The reaction mixture was shaken well and centrifuged at

12000 rpm for 30 min. at 4 C. For electrophoresis 100 ul aliquot of the supernatant was mixed with 50 ul 3X Laemmli loading buffer (50 mM Tris, pH 6.8, 10% glycerol, 6 % sodium dodecylsulpahate SDS, 100 mM dithiothreitol, as a reducing agent and 0.01% bromophenol blue). The sample was then boiled for 3 min. in boiling water bath, and centrifuged at 12000 rpm for 5 min. at room temperature then 20 ul aliquot of the supernatant was subjected for electrophoresis on 7.5% SDS min. gel plate according to the method (Laemmli, 1970), at the end of electrophoresis the gel plate/slap was fixed in a mixture of 40%methanol and 10% acetic acid for 15 min. then stained for protein with 0.25% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid for 30 min. then destained with 30% methanol and 10% acetic acid for 15 min. and subjected to vacuum drying and autoradiography by exposing to Kodak X-OMAT AR film for 10 days at -20 C. The molecular mass of radioactive cross-linked protein bands were estimated by comparison to the migration of high molecular weight protein markers containing myosin (205 KDa.), B-galactase (116 KDa.), Phosphorylase b (97 KDa.), bovine serum albumin (66 KDa.), ovalbumin (45 KDa.) and carbonic anhydrase (29 KDa.).

RESULTS AND DISCUSSION

Figure (1) shows the purity and characteristic morphology of isolated endometrial stromal cells as it displays a typical fusiform and a fibroblast-like phenotype of these cells. From histological point of view the cells show a positive staining with vimentin and exhibit no staining for cytokeratin. These morphological characters and histological staining confirm the purity of endometrial stromal cells in the monolayer culture and exclude any contamination with epithelial cells of uterine tissues.

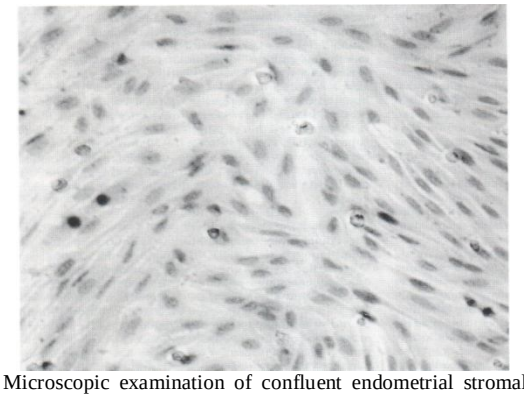

Fig. 1: Microscopic examination of confluent endometrial stromal cells in monolayer culture, negative staining with cytokeratin, passage 2 magnification 400x.

This study indicated the increase of proliferation rate with increasing the concentrations of EGF in a dose-dependent fashion, where the growth response curve reached the plateau level by 10 nM EGF with a proliferation rate of about 150% of the control (Figure 2).

The radiolegand IGF-I was used in the binding studies to investigate and confirm the presence of IGF-I binding component in the reconstituted conditioned medium of stromal cells. Figure (3) shows clearly an increase of the bound radiolegand IGF-I with increasing the concentration of protein fractions of the reconstituted conditioned medium. This proportional relationship of the bouneded radiolegand IGF-I and protein contents confirms however, the presence of high affinity binding components in the conditioned medium of stromal cells. These binding component are in fact a saturable ones as shown in Figure (4).

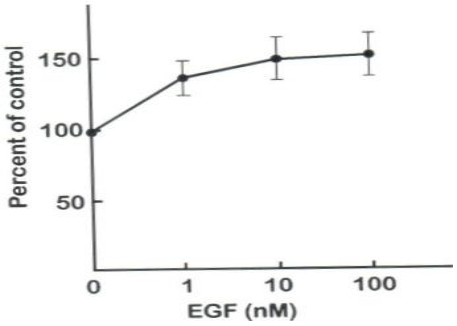

Fig. 2: Growth response curve of pure endometrial stromal cells in a monolayer culture , stimulated with EGF (1, 10, 100 nM) values represent means + SD of three parallel culture.

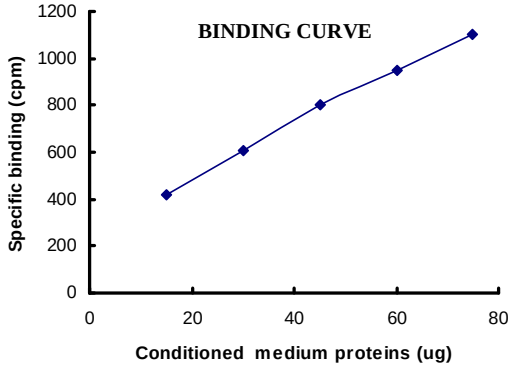

Fig. 3: Specific binding of radiolabelled IGF-I to various concentration of stromal cells conditioned medium proteins. Non-specific binding was less than 10%of the total binding and subtracted from all points.

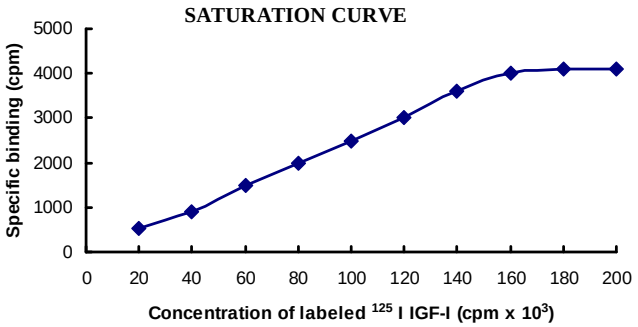

Fig. 4: Saturation of conditioned medium proteins secreted with endometrial stromal cells with radiolabeled IGF-I. Non-specific binding was less than 10% and subtracted from all points.

The saturation of these components with the radiolegend has been studied by incubating 30 ug conditioned medium proteins with serial concentrations of radiolegend IGF-I (20000 – 200000cpm) . Figure (4) shows an increase of the specific binding activity with increasing the radiolegend concentrations till it reach a platue level at 140000 cpm. This saturation curve confirms however, that the

binding site of the secreted components in the condition medium is a saturable one and have a high affinity to the radiolegend IGF-I.

The nature and apparent molecular masses of the radiolegend IGF-I binding components detected in the reconstituted stromal cells conditioned medium has been investigated by electrophoresis and affinity cross-linking technique. The autoradiogram in Figure 5 shows three well separated radiolegend protein bands. In comparison to the migration of the high molecular weight protein markers visualized by Coomassie stain, it was clear that these radiolegend bands have a molecular an approximate weights of about 31, 36, 45 KDa. that may correspond to the IGFBP-1, IGFBP-2 and IGFBP-3, the intensities of these radiolabeled bands in lanes A of the autoradiogram have been semi-quantified by scanning. Figure (6)shows an observable variation in the band intensity, where IGFBP-3 is the most intense band and IGFBP-1 is the less intense one and the band intensity order is IGFBP-3 >IGFBP-2 >IGFBP-1.

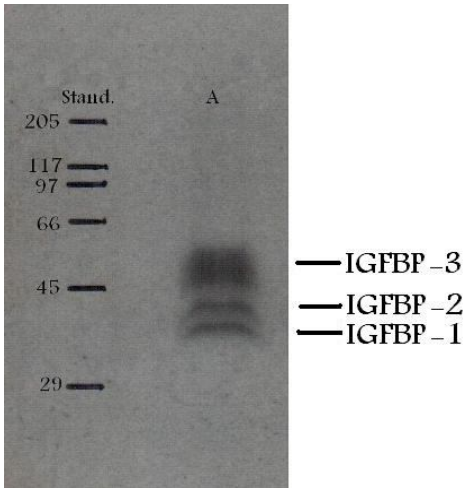

Fig. 5: Affinity cross-linking and autoradiography of radiolabeled IGF-I covalently bounded with conditioned medium proteins secrected by stromal cell culture (Lane A 20 ul).

Endometrium is the lining layer of uterine cavity, it is an epithial-mesenchymal structure whose inner most layer is the stromal cells. Stromal cells layer is the fast growing and proliferative layer of endometrium structure. The biological function of this layer is to provide a receptive endometrium. This layer undergoes a regular cyclic changes proliferation, differentiation and decidualization essential for blastocyst implantation upon its arrival to uterus and further successful embryo development. Steroid sex hormones, IGFs peptides and other growth factors are believed to be involved in these regular cyclic changes either directly or in n autocrine/paracrine manner, The action of the high potent mitogenic IGFs peptides (IGF-I and IGF-II) is thought to be mediated by their nominative receptors on the cell surface IGF-I receptor and IGF-II receptors. This study presents evidences that stromal cells in culture secrete types of proteins that exhibit a high affinity for binding IGFs peptides as identified by specific binding study in Figure (3) where there is a clear direct correlation between conditioned medium protein concentrations and IGF-I specific binding, this relation indicates however, that the stromal cell conditioned medium contains a high specific binding component for IGF-I. This binding components was found to be saturable one as shown in Figure (4) where the specific binding of these component increases with increasing the radiolabeled IGF-I till it comes to platue level , which means that by this level the binding sites of these component is already saturated with the radiolabeled IGF-I.

The nature and molecular masses of these binding component have been characterized with SDS-Page electrophoresis and affinity cross-linking studies as shown in Figure (5) where, the autoradiogram shows a well resolved three successive radiolebeled bands with different molecular masses. The masses of these bands have been determined by comparison to the migration of the high molecular weight proteins markers stained with Coomassie blue The masses have been identified to correspond to IGFBP-I (31 KDa,), IGFBP-2 (36 KDa.) and IGFBP-3 (45 KDa.). These results are in agreement with previous immunoradiometric assay that confirms the secretion of IGFBP-1, GFBP-2 and IGFBP-3 in stromal cell culture (Liu *et al.*, 1995). Immunohistochemical analysis and immunostaining is another technique that identified the presence of IGFBP-1, IGFBP-2 and IGFBP-3 in decidualized stromal cells in implantation site by mouse (Damario *et al.*, 1998). Both Immunoradiometric analysis and immunostaining are also additional proofs that confirm our data.

The intensity of the three radiolabeled bands in our chromatogram have been semiquantified by scanning as shown in Figure 6 where, IGFBP-3 is the most intense band and IGFBP-1 is the less one and the band intensity order was IGFBP-3 > IGFBP-2 > IGFBP-1 This indicates that the stromal cells secretes these IGFBPs with varying amounts and this proves the previous immunoassay findings that IGFBP-3 is the most abundant circulating IGFBPs (Blum *et al.*, 1993) .

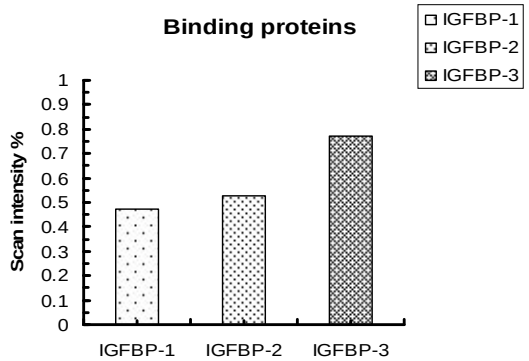

Fig. 6: Scanning of band intensities of the well resolved radiolabeled bands of IGFBP-1, IGFBP-2 and IGFBP-3.

The secretion of IGFBP-1 by predecidualized/decidualized endometrial stromal cells in the late secretory phase endometrium and pregnancy decidua (Rutanen, 1998), as well as the major serection of IGFBPs by human decidualized endometrium in pregnancy (Bell, 1988) may support the view that these IGFBPs may regulate and control the action of IGFs peptides at the

embryo-endometrium interface. The unopposed mitogenic action of IGF-1 due to the absence of IGFBP-1 noticed and characterized all endometrium cancer patients may lead to uncontrolled endometrium proliferation and favor the development of endometrium cancer (Rutanen, 1998). This comfrims the biological importance of IGFBPs in avoiding sever diseases.

This study presents clear evidences that cultured human endometrial stromal cells secrete types of binding proteins identified as IGFBP-1, IGFBP-2 and IGFBP-3 and these proteins exhibit high affinity for the mitogenic peptides IGF-1, with this high affinity, these proteins could control the delivery and bioavailability of the IGF-1 peptides to type-1 IGF-1 receptor thereby, modulating their biological action.

The IGFBP-1, IGFBP-2 and IGFBP-3 secreted by human endometrial stromal cells are considered as a member of the well known IGF-system/IGF-axis that includes IGFs peptides, IGF-receptors, IGFBPs and IGFBP-proteases. Our data and other have confirmed the presence and secretion of these IGFBPs by the functional layer of endometrium /stromal cells, and this support the view that these IGFBPs may participate in preparing endometrium receptivity and blastocyst implantation beside the well known crucial and determinant role of female steroid hormones. So it will be interesting to study the effect of estrogen and progesterone on IGFBPs production by human endometrial stromal cells to investigate the molecular interplay between members of IGF-axis and female steroid hormones on endometrium receptivity and essentiality for embryo development.

ACKNOWLEDGEMENT

This study was supported by the Wilhelm Sander Stifftung. We sincerely thank Prof. Dr. Haering for his help in scanning the autoradiogram and Miss Fileki for her skilled technical assistance in preparing stromal cultures.

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How to cite this article:

Gamal A.M. Singer, Thomas Strowitzki, Jihan Hussein, Fatma Oraby. Identification, Isolation and Characterization of IGF Binding Proteins as a Secretable Proteins of Human Endometrial Stromal Cell Culture. J App Pharm Sci, 2014; 4 (06): 001-008.