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Evidence that α5β1 Integrins Mediate Leydig Cell Binding to Fibronectin and Enhance Leydig Cell Proliferation Stimulated by a Sertoli Cell-Secreted Mitogenic Factor In Vitro

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We reported previously that coculture of immature rat Sertoli cells with Leydig cells or the addition of a concentrate from Sertoli cell-conditioned medium (SCCM) stimulated Leydig cell [^{3}H]-thymidine incorporation, increased cell number, and altered Leydig cell morphology (Wu and Murono, 1994). In the present studies, the effect of various extracellular matrix proteins on immature Leydig cell binding, proliferation and response to SCCM concentrate was investigated. Pretreatment of culture wells with 50 μg/mL collagen I or 10 μg/mL laminin inhibited Leydig cell binding to culture wells about 95 and 89%, respectively; however, 5 μg/mL fibronectin did not change the level of attachment. The binding of Leydig cells to fibronectin was reduced by antifibronectin or -β1 integrin antibodies (66 and 91%, respectively). Treatment of culture wells with five or 50 μg/mL fibronectin alone increased [^{3}H]thymidine incorporation about twofold. When Leydig cells were cultured in wells precoated with increasing concentrations of fibronectin and then treated with SCCM concentrate for 2 d, [^{3}H]-thymidine incorporation increased progressively with the concentration of fibronectin, beyond the levels observed with SCCM concentrate alone. This response was associated with increases in both Leydig cell number and labeling indices. When Leydig cells were cultured on fibronectin, their numbers increased by 3.7- and 5.1-fold following treatment with SCCM concentrates or coculture for 6 d, respectively; whereas, they increased 2.6- and 3.9-fold, respectively, when cultured on plastic. Labeling indices of Leydig cells cultured on plastic for 2 d and treated with SCCM or cocultured were 6.9 and 11.9%, respectively, while labeling indices of Leydig cells grown on fibronectin increased further to 17.6 and 26.3%, respectively. α5β1 integrin complexes and α5 integrin mRNA were expressed in Leydig cells, suggesting that binding to fibronectin may be mediated by α5β1 integrins, a fibronectin receptor. These results suggest that Leydig cell proliferation stimulated by a Sertoli cell-secreted mitogenic factor(s) is enhanced by Leydig cell binding fibronectin, and that this binding may be mediated by α5β1 integrins.

Key Words: Leydig cell; Sertoli cell; mitogen; fibronectin; α5β1 integrins; proliferation.

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Introduction

The regulation of Leydig cell proliferation during sexual, maturation is poorly understood. Previous studies have shown that disruption or damage to the seminiferous tubules causes Leydig cell hypertrophy and hyperplasia, suggesting that Sertoli cells may secrete a mitogenic factor that stimulates Leydig cell proliferation (Aoki and Fawcett 1978; Kerr et al., 1979; Rich and de Kretser, 1979; Risbridger et al., 1981). We have previously demonstrated that coculture of immature rat Sertoli cells with Leydig cells stimulated Leydig cell [^{3}H]thymidine incorporation by 19-fold, increased cell number by 3.9-fold, and dramatically altered the morphology of Leydig cells (Wu and Murono, 1994). The addition of a concentrate from Sertoli cell-conditioned medium (SCCM) to cultured Leydig cells mimicked these biological effects. Partial characterization of the mitogenic factor(s) in SCCM suggested that it is a protein(s) with a molecular weight larger than 10 kDa, that differs in molecular weight, stability, and other characteristics from all previously reported Sertoli cell produced or expressed growth factors.

Extracellular factors determine whether a quiescent cell begins to proliferate and a normal proliferating cell continues to cycle (Pardee, 1989). These factors switch the intracellular machinery between quiescence and G_1 phase. For

many peptide growth factors, their binding to cell surface receptors activates intracellular second messenger pathways that result in DNA synthesis and replication (Hill, 1989). The mitogenic effects of soluble growth factors through binding to their receptors on the cell surface have been reported previously; however, the mechanisms by which extracellular matrix (ECM) molecules modulate the proliferation of cells are not as well studied. A growing body of evidence suggests that ECM proteins influence migration, differentiation, and growth of cells through binding to their receptors, the major group of which are known as integrins (Guan et al., 1991; Adams and Watt, 1993). Binding to integrins generates intracellular signals, such as tyrosine phosphorylation of focal adhesion kinase (FAK, $pp125^{FAK}$) (Burridge et al., 1992; Guan and Shalloway, 1992; Hanks et al., 1992; Kornberg et al., 1992), stimulation of inositol lipid synthesis and enhancement of platelet-derived growth factor (PDGF)-induced inositol lipid breakdown (McNamee et al., 1993), elevation of intracellular Ca^{2+} (Ng-Sikorski et al., 1991), and activation of protein kinase C (Vuori and Ruoslahti, 1993). These intracellular signals presumably mediate the biological effects between integrins and the targeted intracellular sites, although the exact mechanisms are still unknown.

Fibronectin promotes cell growth by binding to integrins on the cell surface (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Ingber et al., 1990). Moreover, it enhances growth factor-stimulated cell proliferation (Ingber et al., 19,87, 1990; Davis et al., 1990). Peritubular cells, in close proximity to Leydig cells, produce fibronectin and collagen I (Tung et al., 1884; Skinner et al., 1985, 1989). Immunocytochemical staining showed that collagen I and fibronectin were localized in the interstitium of rat and human testis (Hadley and Dym, 1987; Santamaria et al., 1990); however, whether they influence Leydig cell function is not known. In the present studies, the ability of immature Leydig cells to bind to different ECM proteins in culture was investigated. Fibronectin was found to facilitate binding, whereas collagen I or laminin reduced attachment of Leydig cells. The basis for this attachment and the possible role of fibronectin in mediating Leydig cell proliferation by Sertoli cell-secreted mitogenic factor(s) were studied.

Materials and Methods

Materials and Animals

Collagenase (type I, ~200 U/mg), penicillin G, streptomycin, N-2-hydroxyethyl piperazine-N'2-ethanesulfonic acid (HEPES) buffer, Ham's F-12 nutrient mixture (F12) and bovine serum albumin (BSA, RIA grade), Triton X-100, $MgCl_2$, $CaCl_2$, Tris, phenylmethylsulfonyl fluoride (PMSF), aprotinin, pepstatin, leupeptin, deoxycholate, sodium dodecyl sulfate (SDS), and ethidium bromide were from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), $NaHCO_3$, Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS), and Taq DNA polymerase were from GIBCO BRL (Grand Island, NY). Percoll and protein A-Sepharose 4B were from Pharmacia Co. (Piscataway, NJ). [Methyl-^{3}H]-thymidine (25 Ci/mmol) was from Amersham Life Science (Arlington Heights, IL). NaOH, acetic acid, methanol and trichloroacetic acid (TCA) were from Fisher Scientific (Pittsburgh, PA). UniverSol and soybean trypsin inhibitor (SBTI) were from ICN Biochemicals (Cleveland, Ohio). Collagen I (Vitrogen 100 purified Collagen) was from Celtrix Pharmaceuticals (Santa Clara, CA). Laminin was purified from the EHS tumor maintained in mice by previously published methods (Kleinman et al., 1982). Fibronectin was purified by gelatin-sepharose affinity chromatography from rat serum (Miekka et al., 1982). Anti-β1 integrin and preimmune IgGs have been described previously (Terracio et al., 1989, 1991; Hilenski et al., 1991). Mono-specific anti-α1 and -α5 integrin IgGs, produced against the cytoplasmic domains of α1 and α5 integrin chains, were from Chemicon (Temecula, CA). Antifibronectin IgGs have been described previously (Lundgren et al., 1988). GeneAmp RNA PCR kit was from Perkin-Elmer (Norwalk, CT). Stratascript RT and random primers (Stratagene Prime IT II) were from Stratagene (La Jolla, CA). Primers for rat glyceraldehyde 3-phosphate dehydrogenase were from Clontech (Palo Alto, CA). pGEM DNA size standard was from Promega (Madison, WI). Giemsa stain was from Fluka Chemical Corp. (Ronkonkoma, NY). Emulsion, developer D-19, and fixer were from Eastman Kodak Co. (Rochester, NY). Centricon-10 was from Amicon Inc. (Beverly, MA). The 100-mm-diameter culture dishes, 35-mm-diameter 6-well and 16-mm-diameter 24-well culture plates, and 24-mm-diameter Transwell tissue culture treated inserts were from Costar (Cambridge, MA).

Charles-Dawley rats were purchased from Charles River Laboratories (Raleigh, NC). Animals were maintained in an AAALAC-accredited facility in compliance with the Guide for the Care and Use of Laboratory Animals. All animal protocols were reviewed and approved by the local animal studies committee. Animals were first rendered unconscious in a saturated CO_2 chamber, then killed by decapitation between 0800 and 0900 h. Testes were excised and placed in an ice bath prior to dissociation with collagenase.

Leydig Cell Isolation

Leydig cells were isolated from 25-d-old rats following the multistep procedure described for adults rats (Klinefelter et al., 1987), but that we have described for immature animals (Murono et al., 1992). Briefly, decapsulated testes were dispersed in 50 U/mL collagenase, and the interstitial cells were fractionated using a Beckman elutriator at a pump speed of 16 mL/min and a rotor speed of 2000 rpm. Cells that were retained at these settings were layered over a 60% self-generating Percoll gradient, and centrifuged at 27,000*g*

for 1 h. Cells localizing at a density of 1.068 g/mL or higher were saved as the Leydig cell fraction. Histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD) was used to assess the purity of these cells, which represented 94–96% Leydig cells (Wu and Murono, 1994).

Sertoli Cell Isolation

Sertoli cells from 25-d-old rats were isolated according to Rich et al. (1983) with some modifications (Wu and Murono, 1994). In brief, decapsulated testes were digested with 50 U/mL collagenase for 20 min with agitation (80 cpm) at 37°C. After incubation, the tubules were washed three times with 30 mL fresh medium and allowed to settle by unit gravity for 10 min between washes. The tubules were digested with collagenase (50 U/mL, 30 min at 37°C) a second time. After incubation, the cellular suspension was brought up to 30 mL by addition of fresh medium, and the Sertoli cell aggregates were isolated by allowing the cells to settle by unit gravity for 15 min. The Sertoli cell aggregates were resuspended in fresh medium, and this washing step was repeated. The supernatant was removed, and 10 mL of fresh medium was added. Sertoli cell aggregates were dispersed further by repeated pipeting using an automatic pipet. Additional 30 mL of medium was added and the dispersed cells were passed through a four-layer gauze filter. The Sertoli cell aggregates were washed three more times with 30 mL fresh medium and allowed to settle by unit gravity for 20 min between washes. Each Sertoli cell aggregate contained 5–15 cells. After 2 d of culture, cells were washed with fresh medium to remove unattached cells and cellular debris. This yielded a preparation with a purity of 94±2% Sertoli cells as reported previously (Wu and Murono, 1994).

Sertoli-Leydig Cell Coculture or Treatment of Leydig Cells with SCCM

Coculture of Leydig and Sertoli cells or treatment of Leydig cells with SCCM were as described previously (Wu and Murono, 1994). Leydig cells were plated into 35-mm-diameter 6-well culture plates, with or without coating of 5 μg/mL fibronectin, at a density of 2×10^5/well, and Sertoli cells were pipette into 24-mm-diameter Transwell inserts at a density of 10^5 aggregates/insert. Cells were cultured separately at 37°C and washed with fresh media to remove cellular debris and unattached cells 48 h after plating. Leydig cells were cocultured with Sertoli cells or treated with 10 μL of SCCM concentrate for 48–120 h. SCCM concentrate was prepared as described previously (Wu and Murono, 1994). The Sertoli cell inserts were removed and [^{3}H]-thymidine incorporation, labeling index, morphology, and number of Leydig cells were examined as described previously (Wu and Murono, 1994).

Cell-Attachment Assay

16-mm-diameter 24-well plates were uncoated, coated with 5 μg/mL fibronectin or 10 μg/mL laminin at 4°C overnight, or 50 μg/mL collagen at 4°C for 1 h. Wells were rinsed twice with culture medium, and 5×10^4 Leydig cells were plated into the wells. In some experiments, antifibronectin IgGs or -β1 integrin IgGs were added to block Leydig cell binding to fibronectin. In these experiments, wells coated with fibronectin were incubated with 2% BSA at 37°C for 1 h. For the experiments using antifibronectin IgGs, wells were then incubated with culture medium or 250 μg/mL antifibronectin IgGs at 37°C for 1 h and 5×10^4 Leydig cells were plated into the wells. For the experiments using anti-β1 integrin IgGs, Leydig cells were incubated with culture medium or 500 μg/mL anti-β1 integrin IgGs in culture medium at 37°C for 1 h, and 5×10^4 cells were plated to each well. For both of the antibody experiments, preimmune IgGs (rabbit) were used at the same concentrations as control. To assay cellular attachment, cells were cultured for 24 h. They were washed three times with PBS, fixed with methanol, stained with Giemsa, and examined under a phase-contrast microscope. At least 200 fields (200× magnification) were randomly selected and counted for each well.

Immunoprecipitation of β1 Integrin Subunit in Cultured Leydig Cells

Leydig cells were cultured for two days and washed with fresh culture medium. Integrin chains present on the surface of cultured cells were determined using methods we have described previously (Gullberg et al., 1989; Terracio et al., 1991). Cells were surface-labeled by iodination with ^{125}I (Gullberg et al., 1989). Next, the cells were washed with PBS and solubilized in buffer containing 1% Triton X-100, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10 m*M* Tris (pH 8.0) and protease inhibitors (1 m*M* PMSF, 1% aprotinin, 1 μg/mL pepstatin A, and 2 m*M* leupeptin). Cells were centrifuged at 15,000*g* for 30 min, and the supernatant was saved. Equal radioactive counts of the supernatants were used for immunoprecipitation. Preimmune IgGs (100 μg/mL) were added to the supernatant and samples were incubated for 4 h. This was followed by the addition of 100 μL protein A-Sepharose 4B (50% slurry in PBS), and samples were incubated for an additional 1 h. Following centrifugation at 15,000*g*, the supernatant was incubated with immune IgGs against the integrin β1 (Terracio et al., 1991), α1 or α5 chain (100 μg/mL) overnight. Protein A-Sepharose was added as described above (Terracio et al., 1991) and incubation continued for 1 h. Immunoprecipitated proteins were rinsed four times in buffer containing 1% Triton X-100, 0.5*M* NaCl, 1 m*M* $CaCl_2$, and 10 m*M* Tris (pH 7.4), twice in buffer containing 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$ and 0.1*M* NaCl (pH 8.0) and once in PBS. Immunoprecipitates were isolated by centrifugation at 15,000*g*, and the pellets were resuspended in SDS-PAGE sample buffer. The sample was boiled and subjected to SDS-PAGE and autoradiography as previously described (Gullberg et al., 1989). Identification of α chains

was verified by immunoprecipitation with α1 or α5 integrin antibodies as described previously in cardiac myocytes (Terracio et al., 1991).

Identification of α5 Integrin mRNA

Reverse transcriptase (RT) polymerase chain reaction (PCR) was performed as described by Burgess et al. (1994) to determine whether mRNA for α5 integrin was expressed in Leydig cells. Total RNA was isolated from freshly isolated Leydig cells using the one-step guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). RT-PCR was performed using 1 μg of total RNA per reaction. Briefly, Stratascript RT and random primers (Stratagene Prime IT II) were used for cDNA synthesis from RNA. This cDNA was amplified using Taq DNA polymerase and primers for mouse α5 integrin (Holers et al., 1989). Specifically, cDNA sequences nt 97-118 (CTGAACCTGACATTCCATGCCC) and nt 500-521 (TGGTCTTGAGGATTCCAGTCGC) were used. The primer detection software (Clontech [Palo Alto, CA]) was used to select the α5 integrin primers for this experiment. These primers were synthesized by the Oligonucleotide Synthesis Facility at the University of South Carolina (Institution for Biological and Research Technology, Columbia, SC). Primers for rat glyceraldehyde 3-phosphate dehydrogenase were used as a positive control. Thermal cycles were carried out for 32 cycles at 95°C for 1.5 min, 50°C for 1.5 min, 72°C for 2 min, with a single final 72°C incubation for 10 min. Amplified DNA was separated on 2% agarose gels along with pGEM DNA-size standards for reference. Gels were stained with ethidium bromide and photographed against UV light with a Polaroid camera.

Statistics

Each experiment was performed at least three times, each with similar results. The means of treatment groups were compared using analysis of variance (Tukey's multiple comparison procedure with contrasts). The means of treatment groups with two factors were compared using two-way analysis of variance (Tukey's multiple comparison procedure with contrasts). A p value of 0.05 or less was considered statistically significant.

Results

Effect of ECM Proteins on Leydig Cell Attachment

Leydig cell adhesion to ECM proteins was examined by coating culture wells with these proteins, then quantitating the number of cells that remained firmly attached to each well following two d of culture. Treatment of culture wells with 50 μg/mL collagen I or 10 μg/mL laminin inhibited immature Leydig cell binding about 95% and 89%, respectively, when compared to control (Table 1). Treatment of culture wells with 5 μg/mL fibronectin did not significantly alter the attachment of Leydig cells when compared to control.

Table 1
The Effect of Extracellular Matrix Proteins on Leydig Cell Attachment[a]

Treatment group	Cells/100 fields, 200×	% of control
Control	2966 ± 404	100.0
5 μg/mL fibronectin	3249 ± 340	109.5 ± 11.5
10 μg/mL laminin	327 ± 30[b]	11.0 ± 1.0
50 μg/mL collagen	144 ± 41[b]	4.9 ± 1.3

[a]2×10^5 Leydig cells from 25-d-old rats were plated to 35-mm-diameter culture wells without coating (control) or coated with collagen, laminin, or fibronectin. They were cultured for 48 h, then washed with fresh culture medium to remove unattached cells. Attached cells were counted under phase-contrast microscope. Each treatment group represents the mean ± SE of three separate cultures. [b]Significantly different from control ($p < 0.0001$).

Table 2
The Effect of Antifibronectin or -β1 Integrin Antibodies on Leydig Cell Attachment[a]

Treatment group	Cells/100 fields, 200×	% of control
Control	2549 ± 131	100.0
250 μg/mL preimmune IgGs	2648 ± 311[b]	103.4 ± 12.2
250 μg/mL antiFN IgGs	863 ± 205[c]	33.9 ± 8.0
500 μg/mL preimmune IgGs	2686 ± 324[b]	105.4 ± 12.7
500 μg/mL antiβ1 IgGs	238 ± 44[c]	9.3 ± 1.7

[a]Culture plates were coated with 5 μg/mL fibronectin. Wells were rinsed twice with culture medium and incubated with 2% BSA at 37°C for 1 h. Leydig cells were incubated with culture medium (control), 250 μg/mL antifibronectin (FN) or 500 μg/mL anti-β1 integrin IgGs in culture medium at 37°C for 1 h, and 5×10^4 cells were plated to each well. Preimmune IgGs (rabbit) were used at both concentrations as controls. All the cells were cultured for 24 h to enable them to attach. Cells were washed three times with PBS, fixed with methanol, and stained with Giemsa. Attachment of cells was assessed with an inverted phase-contrast microscope. Each number represents the mean ± SE of three separate cultures.
[b]Not significantly different from control ($p > 0.05$).
[c]Significantly different from control ($p < 0.0001$).

Blockage of Leydig Cell Binding to Fibronectin by Fibronectin or β1 Integrin Antibodies

To examine whether Leydig cell binding to fibronectin is specific and mediated by the fibronectin receptor, α5β1 integrin, antifibronectin or -β1 integrin IgGs were used to block Leydig cell binding to fibronectin. Treatment of fibronectin-coated wells with 250 μg/mL antifibronectin IgGs blocked Leydig cell binding about 66% (Table 2). Treatment of Leydig cells with 500 μg/mL anti-β1 integrin IgGs

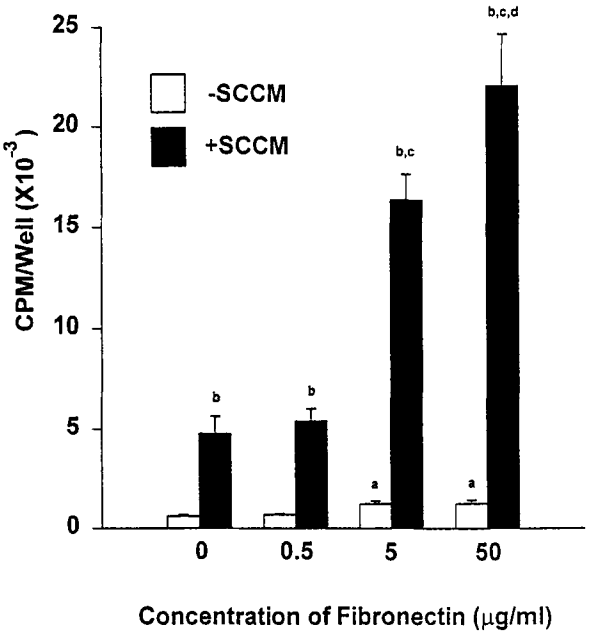

Fig. 1. The synergistic effect of the Sertoli c ell-secreted mitogenic factor(s) and fibronectin on Leydig cell [^{3}H]-thymidine. Leydig cells were plated into 16-mm-diameter 24-well plates, uncoated or coated with increasing concentrations of fibronectin (0.5, 5, and 50 μg/mL), at 5×10^4 cells/well. They were untreated or treated with 10 μL of the SCCM concentrate in 0.5 mL culture medium 48 h after plating. Leydig cell [^{3}H]-thymidine incorporation was performed 2 d after treatment. [a]Significantly different from Leydig cells cultured on plastic or 0.5 μg/mL fibronectin ($p < 0.005$). [b]Significantly different from their controls ($p < 0.005$). [c]Significantly different from cells cultured on plastic or 0.5 μg/mL fibronectin and treated with SCCM ($p < 0.0001$). [d]Significantly different from cells cultured on 5 μg/mL fibronectin and treated with SCCM ($p < 0.0001$).

blocked Leydig cell binding to fibronectin about 91%. Comparable concentrations of preimmune IgGs had no effect on Leydig cell attachment. These results suggested that Leydig cell binding to fibronectin was specific and mediated by cell surface α5β1 integrins.

Effect of Fibronectin and SCCM Concentrate on Leydig Cell [^{3}H]-Thymidine Incorporation

Pretreatment of culture wells with 0.5 μg/mL fibronectin alone had no effect on Leydig cell [^{3}H]-thymidine incorporation; however, 5 and 50 μg/mL fibronectin increased [^{3}H]thymidine incorporation about twofold over 2 d of culture (Fig. 1). When Leydig cells, cultured on wells precoated with various concentrations of fibronectin, were treated with SCCM concentrate for 2 d, [^{3}H]-thymidine incorporation increased progressively with the concentration of fibronectin, beyond the levels stimulated by SCCM concentrate alone. [^{3}H]thymidine incorporation of Leydig cells cultured on 50 μg/mL fibronectin-coated wells and treated with SCCM concentrate was 4.6 times higher than that of Leydig cells cultured on plastic and treated with SCCM concentrate. These results suggest a synergism between fibronectin and SCCM concentrate.

Table 3
The Effect of Fibronectin and Sertoli Cell-Secreted Mitogenic Factor(s) on Leydig Cell Number[a]

	–Fibronectin		+Fibronectin	
Treatment group	Cells/ 100 fields, 200×	% of control[b]	Cells/ 100 fields 200×	% of control[b]
Control	2812 ± 415	100	3725 ± 768[c]	132 ± 27
SCCM	6268 ± 568[d]	255 ± 23	10,482 ± 1792[e,f]	373 ± 64
SC	9500 ± 879[e]	386 ±.36	14,367 ± 2246[e,f]	511 ± 80

[a]2×10^5 Leydig cells from 25-d-old rats were plated to 35-mm-diameter culture wells without (-fibronectin) or precoated with 5 μg/mL fibronectin (+fibronectin). They were cultured for 48 h, then washed with fresh culture medium to remove unattached cells. Cells were treated with SCCM concentrate or cocultured with Sertoli cells for six days. They were washed, fixed with methanol, and stained with Giemsa. Control: Leydig cells cultured alone; SCCM: Leydig cells cultured alone but treated with 10 μL SCCM concentrate; SC: Leydig cells cocultured with Sertoli cells. Each treatment group represents the mean ± SE of three separate cultures.

[b]Percentages of control Leydig cells cultured alone on plastic.

[c]Not significantly different from control without fibronectin ($p > 0.05$).

[d]Significantly different from its control ($p < 0.05$).

[e]Significantly different from their respective control ($p < 0.0005$).

[f]Significantly different from cells cultured on plastic with the same treatment ($p < 0.005$).

Effect of Fibronectin on Leydig Cell Morphology, Number, and Labeling Index (LI)

Dramatic morphological changes in Leydig cells from a rounded outline to a fibroblastic appearance were observed after coculture or treatment with SCCM concentrate in the previous studies (Wu and Murono, 1994). Leydig cells cultured on 5 μg/mL fibronectin-treated wells spread modestly over six days of culture. However, cells grown on the fibronectin and treated with SCCM concentrate appeared to spread much faster and were far more extended (not shown).

Leydig cells cultured on plastic or 5 μg/mL fibronectin-coated wells were treated with SCCM concentrate or cocultured with Sertoli cells for six days to examine whether there was a synergistic effect between the mitogenic factor and fibronectin on cell number. Leydig cells cultured on plastic treated with SCCM concentrate or cocultured with Sertoli cells increased 2.6- and 3.9-fold, respectively, as reported previously (Wu and Murono, 1994). The present studies demonstrated a further increase in Leydig cell number from the combined effects of fibronectin and SCCM concentrate. Under these conditions, SCCM concentrate and coculture of Leydig and Sertoli cells increased Leydig cell number by 3.7- and 5.1-fold, respectively (Table 3).

Leydig cell-labeling indices were also examined following treatment of Leydig cells cultured on plastic or fibronectin with SCCM concentrate or coculture. After 2 d

Table 4
The Effect of Fibronectin and the Sertoli Cell-Secreted Mitogenic Factor(s) on [^{3}H]Thymidine Labeling Index of Immature Leydig Cells[a]

Treatment group	Control	SCCM	SC
-fibronectin	0.4 + 0.1	6.9 ± 1.5[c]	11.8 ± 2.3[c,d]
+fibronectin	0.9 + 0.2[b]	17.6 ± 3.6[c]	26.3 + 2.9[c,d]

[a]2 × 10^5 Leydig cells from 25-d-old rats were plated to 35-mm-diameter culture wells without (-fibronectin) or precoated with 5 μg/mL fibronectin (+fibronectin). They were cultured for 48 h, then washed with fresh culture medium to remove unattached cells. Then some Leydig cells were treated with SCCM concentrate or cocultured with Sertoli cells for two days. Sertoli cell inserts were removed, cells were washed and 1 mL of fresh medium was added. Leydig cells were incubated with 4 μCi [^{3}H]-thymidine for 24 h and autoradiography and Giemsa staining were performed. Control: Leydig cells cultured alone; SCCM: Leydig cells cultured alone but treated with 10 μL SCCM concentrate; SC: Leydig cells cocultured with Sertoli cells. Each treatment group represents the mean ± SE of three separate cultures.

[b]Not significantly different from control without fibronectin ($p > 0.05$).

[c]Significantly different from their respective controls ($p < 0.005$).

[d]Significantly different from Leydig cells treated with SCCM concentrate in each group ($p < 0.005$).

of treatment, labeling indices of Leydig cells cultured on plastic and treated with SCCM or coculture were 6.9 and 11.9%, respectively, whereas labeling indices of Leydig cells cultured alone was only 0.4% (Table 4). Labeling indices of Leydig cells under the same treatment conditions, but grown on fibronectin increased further, and were 17.6 and 26.3% for SCCM concentrate treatment and coculture, respectively.

Expression of Integrins in Leydig Cells

The previous studies demonstrated that culturing Leydig cells on fibronectin enhanced the mitogenic effects of SCCM. We next examined which integrins are expressed by immature Leydig cells. Cultured Leydig cells were surface labeled with ^{125}I, solubilized, immunoprecipitated using anti-β1 integrin IgGs, and resolved by SDS-PAGE (Fig. 2). The immunoprecipitation of β1 subunit by anti-β1 integrin IgGs and associated α5 subunit was demonstrated by the dark bands in the autoradiograph. There was also a very light band, which indicated α1 subunit associated with β1 subunit, probably representing low levels of α1β1 complexes in Leydig cells or contaminating cells. No other associated integrin subunits were detected by immunoprecipitation. The identification of these integrin chains was confirmed by immunoprecipitation with monospecific IgGs to the α5 or α1 integrin (data not shown), as we have described previously (Terracio et al., 1991).

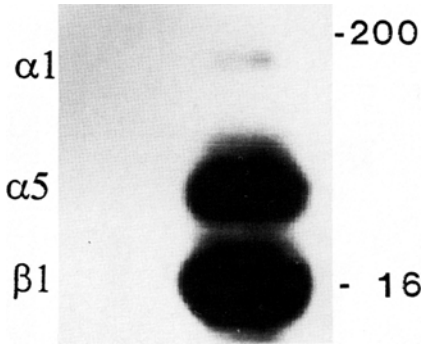

Fig. 2. The expression of integrins in Leydig cells. Immunoprecipitation was performed using anti-β1 integrin IgGs and cell surface labeling with ^{125}I. Preimmune IgGs did not precipitate integrins on cell surface. The immunoprecipitation of β1 subunit (β1) by anti-β1 integrin IgGs and associated α5 subunit (α5) is shown. The light band indicates α1 subunit (α1) associated with β1 subunit. No other associated integrin subunits were detected by immunoprecipitation. Numbers represent kilodaltons.

Expression of Integrin mRNA in Leydig Cells

RT-PCR was used to identify α5 integrin mRNA in Leydig cells. Synthesis of cDNA from Leydig cell mRNA with random primers and subsequent PCR with α5 integrin-specific primers resulted in the amplification of the anticipated DNA product of approx 424-base pair (bp) (Fig. 3, lane 1). Amplification with glyceraldehyde 3-phosphate dehydrogenase primers also resulted in the appropriate size DNA and served as a positive control (Fig. 3, lane 2).

Discussion

We have previously shown that Sertoli cells secrete a mitogenic factor(s) which stimulates proliferation and inhibits steroidogenesis of Leydig cells (Wu and Murono, 1994). In the present studies, we demonstrated that the proliferative effect of the mitogenic factor(s) can be greatly enhanced by fibronectin. In contrast, collagen I and laminin reduced Leydig cell adhesion to culture plates. α5β1 integrins were identified and α5 integrin subunit mRNA was expressed in Leydig cells, suggesting that the effects of fibronectin may be mediated through this classic fibronectin receptor.

ECM proteins have been reported to influence migration, differentiation, and growth of cells through binding to their receptors known as integrins (Guan et al., 1991; Adams and Watt, 1993). It was shown previously that fibronectin enhanced growth factor-stimulated proliferation of T lymphocytes (Davis et al., 1990) and capillary endothelial cells (Ingber et al., 1987; Ingber, 1990). Moreover, it was demonstrated that proliferation proceeded only when fibronectin was present in insoluble form (Davis et al., 1990, Ingber, 1990) and when cellular spreading and nuclear expansion occurred (Ingber, 1990). It has also been shown that fibronectin stimulates growth of fibroblasts (Terranova et al., 1986), and granulosa cells (Morley et al., 1987) that are both of mesenchymal origin, as is the case for Leydig cells.

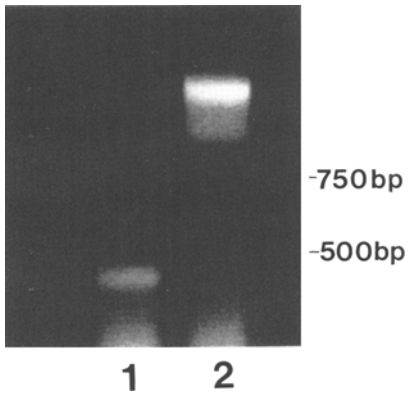

Fig. 3. RT-PCR showing Leydig cell expression of α5 integrin mRNA. Total RNA was isolated from freshly isolated Leydig cells using the one-step guanidinium thiocyanate method. RT-PCR was performed using 1 μg of total RNA per reaction. Stratascript RT and random primers were used for cDNA synthesis from the RNA. The cDNA was amplified using Taq DNA polymerase and primers (nt 97-118: CTGAACCTGACATTCCATGCCC and nt 500-521: TGGTCTTGAGGATTCCAGTCGC) for the mouse α5 integrin. Primers for rat glyceraldehyde 3-phosphate dehydrogenase were used as a positive control. Amplified DNA was separated by 2% agarose gels along with pGEM DNA size standards for reference. The band (424 bp) in lane 1 represents amplified cDNA from α5 integrin mRNA. The band in lane 2 represents amplified cDNA from glyceraldehyde 3-phosphate dehydrogenase mRNA.

We have shown in the present studies that immature rat Leydig cells adhered to fibronectin and plastic equally well; however, they attached to laminin or collagen I poorly. It is not clear whether Leydig cells secrete fibronectin or other ECM components that mediate their binding to plastic. Although Leydig cells attached to both plastic and fibronectin, their morphological appearance following culture on either substrate differed. Leydig cells cultured on plastic did not spread out, whereas Leydig cells plated on fibronectin slowly spread out after several days of culture. However, the time required for this spreading was much slower and far less prominent than that observed following treatment of cells with SCCM concentrate (Wu and Murono, 1994). In contrast, culturing adult Leydig cells on fibronectin-coated wells had only a modest effect on Leydig cell spreading (Vernon et al., 1991). This may suggest that immature and adult Leydig cells bind to or respond differently to fibronectin. Leydig cells grown on fibronectin and treated with SCCM concentrate appeared to spread at a faster rate and to a greater degree, suggesting that fibronectin and the mitogenic factor(s) acted synergistically. Synergistic effects were more clearly quantitated when other endpoints were measured. For example, [^{3}H]-thymidine incorporation of Leydig cells grown on fibronectin without treatment increased slightly over two d of culture, suggesting that fibronectin alone has very limited effect on Leydig cell proliferation. Although a direct proliferating effect of fibronectin was lacking, the growth response of Leydig cells cultured on fibronectin to the mitogenic factor(s) was greatly increased as indicated by increased [^{3}H]-thymidine incorporation, labeling index, and number of Leydig cells. The mechanisms of this synergism are not clear at present. The Sertoli cell-secreted mitogenic factor(s) was previously shown to cause Leydig cell spreading and proliferation on plastic (Wu and Murono, 1994). In the present studies, a faster and more extended spreading of cells and nuclei was observed in Leydig cells grown on fibronectin following treatment with SCCM concentrate, suggesting that cellular and nuclear expansion may be associated with the level of proliferation. Binding of Leydig cells to fibronectin may facilitate the expansion of cells and nuclei, and reorganization of the cytoskeleton and nuclei. These changes are proposed to favor DNA synthesis and to be a prerequisite for cellular entry into the S-phase of the cell cycle (Ingber et al., 1987; Ingber, 1990; Nicolini et al., 1986). The rate of endothelial cell proliferation was tightly coupled to cellular and nuclear expansion (Folkman and Moscona, 1978). Alternatively, fibronectin may act as a competence factor and signal Leydig cell response to a progression factor(s), which is presumably present in SCCM. This mechanism of synergy was proposed by Bitterman et al., (1983) who observed dose-dependent growth stimulation of HFL1 fibroblasts by fibronectin. This effect of fibronectin was enhanced by insulin and alveolar macrophage-derived growth factor, but not by fibroblast growth factor (FGF). It is not clear whether this is true for Leydig cell proliferation stimulated by the mitogenic factor(s) in SCCM. Leydig cell spreading in response to SCCM concentrate was observed in cells cultured on plastic alone; therefore, it is possible that Leydig cells secrete fibronectin to facilitate cell spreading in response to the mitogenic factor(s). Secretion of extracellular matrix proteins by Leydig cells has not been demonstrated previously, although peritubular cells, which are thought to be derived from mesenchymal cells, as is the case for Leydig cells, secrete collagen I (Skinner et al., 1985) and fibronectin (Tung et al., 1884; Skinner et al., 1989).

It is possible that binding of Leydig cells to fibronectin generates intracellular signals that converge with signals generated by mitogenic factor(s). Although the mechanism mediating cellular shape changes are not known, there is evidence that binding of ECM proteins to integrins generates intracellular signals, including tyrosine phosphorylation of FAK (Burridge et al., 1992; Guan and Shalloway, 1992; Hanks et al., 1992; Kornberg et al., 1992), stimulation of inositol lipid synthesis and enhancement of PDGF-induced inositol lipid breakdown (McNamee et al., 1993), elevation of intracellular Ca^{2+} (Ng-Sikorski et al., 1991), and activation of protein kinase C (Vuori and Ruoslahti, 1993). Studies on KB carcinoma cells (Kornberg et al., 1991), and NIH 3T3 fibroblasts (Guan et al., 1991) showed that the crosslinking of integrins or binding of cells on fibronectin induced rapid phosphorylation of several proteins of similar size. These may be related to proteins

that are phosphorylated by treatment of cells with various soluble growth factors (Rees-Jones and Taylor, 1985; Sadoul et al., 1985; Pasquale et al., 1988). The phosphorylation of similar proteins by soluble growth factors and activation of integrins by ECM proteins suggest the existence of convergent signaling pathways, and this may offer a potential explanation for anchorage-dependent cell growth in normal cells (Hynes, 1992).

In the rat, fibronectin disappears from differentiating mesenchymal cells in many organs (Rouslahti et al., 1981), including testicular cord cells from the 13.5-d-old fetus (Pelliniemi et al., 1984). However, fibronectin was identified in the lamina propria and within the interstitium of adult rat and human testes, while collagens I and IV and laminin were limited to the lamina propria (Hadley and Dym, 1987; Santamaria et al., 1990). The functions of these ECM components are not well understood. However, with respect to Leydig cells, the present studies demonstrated that they bind preferentially to fibronectin-coated plastic wells over collagen I- and laminin-coated wells. This selective binding to fibronectin and the growth-promoting effects of fibronectin suggest that Leydig cells may possess mainly fibronectin receptors (integrins). Although the specific functions of SCCM and fibronectin in immature Leydig cells remain to be determined, it is noteworthy that between days 14 and 28 of maturation, the number of rat Leydig cells increase from about 0.4 to 13.4 million per testis (Hardy et al., 1989). Although this increase has been ascribed mainly to differentiation of mesenchymal precursors (Hardy et al., 1989), the present studies suggest that SCCM together with fibronectin also may play a role in this increase.

Integrins are a family of heterodimeric cell surface receptors consisting of an α and β subunit (Hynes, 1992; Juliano and Haskill, 1993). Currently at least 14 distinct α subunits and 8 or more β subunits that can associate in various combinations have been identified (Juliano and Haskill, 1993). Binding specificities of various extracellular matrix proteins are determined by the various α/β associations. The "classic" fibronectin receptor, α5β1, only recognizes a single ligand (Brown and Juliano, 1985). Our present immunoprecipitation results appear to be consistent with these previous results, because α5 integrins were shown to associate with β1 integrins in immature Leydig cells. A very light band representing α1 integrin associated with β1 integrin was observed. α1β1 association is the receptor for both laminin and collagen (Hynes 1992). The presence of this complex is consistent with the present attachment studies that showed that about 11% of plated cells bound to laminin and about 4% to collagen I. Since some of the contaminating cells in our preparation are peritubular cells, it is possible that they possess α1β1 integrins. The type(s) of ECM receptors present in peritubular cells have not been reported. In rat testis, peritubular cells are sandwiched between the two layers of lamina propria that are composed of large amounts of laminin, collagen I and IV (Hadley and Dym, 1987). It is possible that peritubular cells interact with these ECM proteins via receptors such as α1β1 integrins. Alternatively, Leydig cells may express α1β1 integrins at such a low level that they are not able to efficiently mediate Leydig cell binding to laminin and collagen I.

The present studies suggest that fibronectin has a functional role in Leydig cell proliferation. Because fibronectin is only present in the interstitial compartment and Leydig cells can bind to it, but not other ECM proteins such as laminin and collagen I, it is possible that fibronectin contributes to the organization of Leydig cells in the interstitium. It may function as a mediator between Leydig cells and other ECM components, such as the various collagen types (Engvall et al., 1978; Ruoslahti and Engvall, 1980), glycosaminoglycans and fibrin (Yamada et al., 1980).

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References

Adams, J. C. and Watt, F. M. (1993). *Development* **117,** 1183–1198.

Aoki, A. and Fawcett, D. W. (1978). *Biol. Reprod.* **19,** 144–153.

Bitterman, P. B., Rennard, S. I., Adelberg, S., and Crystal, R. G. (1983). *J. Cell Biol.* **97,** 1925–1932.

Brown, P. J., and Juliano, R. L. (1985). *Science* **228,** 1448–1451.

Burgess, M. L., Carver, W. C., Terracio, L., Wilson, S. P., Wilson, M. A., and Borg, T. K. (1994). *Circ. Res.* **74,** 291–298.

Burridge, K., Turner, C., and Romer, L. H. (1992). *J. Cell Biol.* **119,** 893–903.

Chomczynski, P. and Sacchi, N. (1987). *Anal. Biochem.* **162,** 156–159.

Davis, L. S., Oppenheimer-Marks, N., Bednarczyk, J. L., McIntryre, B. W., and Lipsky, P. E. (1990). *J. Immunol.* **145,** 785–793.

Engvall, E., Ruoslahti, E., and Miller, E. J. (1978). *J. Exp. Med.* **147,** 1584–1595.

Folkman, J. and Moscona, A. (1978). *Nature* **273,** 345–349.

Guan, J.-L. and Shalloway, D. (1992). *Nature* **358,** 690–692.

Guan, J. L., Trevithick, J. E., and Hynes, R. O. I (1991). *Cell Regul.* **2,** 951–964.

Gullberg, D., Terracio, L., Borg, T. K., and Rubin, K. (1989). *J. Biol. Chem.* **264,** 12,686–12,694.

Hadley, M. A. and Dym, M. (1987). *Biol. Reprod.* **37,** 1283–1289.

Hanks, S. K., Calalb, M. B., Harper, M. C., and Patel, S. K. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 8487–8491.

Hardy, M. P., Zirkin, B. P., and Ewing, L. L. (1989). *Endocrinology* **124,** 762–770.

Hilenski, L. L., Terracio, L., and Borg, T. K. (1991). *Cell Tissue Res.* **264,** 577–587.

Hill, D. J. (1989). *J. Reprod. Fertil.* **85,** 723–734.

Holers, V. M., Ruff, T. G., Parks, D. L., McDonald, J. A., Ballard, L. L., and Brown, E. J. (1989). *J. Exp. Med.* **169,** 1589–1605.

Hynes, R. O. (1987). *Cell* **48,** 549–554.

Hynes, R. O. (1992). *Cell* **69,** 11–25.

Ingber, D. E. Prusty, D., Frangioni, J. V., Cragoe, Jr., E. J., Lechene, C., and Schwartz, M. A. (1990). *J. Cell Biol.* **110,** 1803–1811.

Ingber, D. E. Madri, J. A., and Folkman, J. (1987). *In Vitro Cell Dev. Biol.* **23,** 387–394.

Ingber, D. E. (1990). *Proc. Natl. Acad. Sci. USA* **87,** 3579–3583.

Juliano, R. L. and Haskill, S. (1993). *J. Cell Biol.* **120,** 577–585.

Kerr, J. B., Rich, K. A., and de Kretser, D. M. (1979). *Biol. Reprod.* **20,** 409–422.

Kleinman, H. K., McCarvey, M. L., Liotta, L. A., Robey, P. G., Tryggvason, K., and Martin, G. R. (1982). *Biochemistry* **21,** 6188–6193.

Klinefelter, G. R., Hall, P. F., and Ewing, L. L. (1987). *Biol. Reprod.* **36,** 769–783.

Kornberg, L., Earp, H. S., Turner, C. E., Prockop, C., and Juliano, R. L. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 8392–8396.

Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992). *J. Biol. Chem.* **267,** 23,439–23,442.

Lundgren, E., Gullberg, D., Rubin, K., Borg, T. K., Terracio, M. J., and Terracio, L. (1988). *J. Cell. Physiol.* **136,** 43–53.

McNamee, H. P., Ingber, D. E., and Schwartz, M. A. (1993). *J. Cell Biol.* **121,** 673–678.

Miekka, S. I., Ingham, K. C., and Menache, D. (1982). *Thromb. Res.* **27,** 1–14.

Morley, P., Armstrong, D. T., and Gore-Longton, R. E. (1987). *J. Cell Physiol.* **132,** 226–236.

Murono, E. P., Washburn, A. L., Goforth, D. P., and Wu, N. (1992). *Mol. Cell. Endocrinol.* **88,** 39–45.

Ng-Sikorski, J., Andersson, R., Patarroyo, M., and Andersson, T. (1991). *Exp. Cell Res.* **195,** 504–508.

Nicolini, C., Belmont, A. S., and Martelli, A. (1986). *Cell Biophys.* **8,** 103–117.

Pardee, A. B. (1989). *Science* **246,** 603–608.

Pasquale, E. B., Mather, P. A., and Singer, S. J. (1988). *J. Cell Physiol.* **137,** 146–156.

Pelliniemi, L. J., Parako, J., Grund, S. K., Frojdman, K., Foidart, J.-M., and Lakkala-Paranko, T. (1984). *Ann. NY Acad. Sci.* **438,** 405–416.

Rees-Jones, R. W. and Taylor, S. I. (1985). *J. Biol. Chem.* **260,** 4461–4467.

Rich, K. A., Kerr, J. B., and de Kretser, D. M. (1979). *Mol. Cell. Endocrinol.* **13,** 123–135.

Rich, K. A. and de Kretser, D. M. (1979). *Intl. J. Androl.* **2,** 343–352.

Rich, K. A., Bardin, C. W., Gunsalus, G. L., and Mather, J. P. (1983). *Endocrinology* **113,** 2284–2293.

Risbridger, G. P., Kerr, J. B., and de Kretser, D. M. (1981). *Biol. Reprod.* **24,** 534–540.

Ruoslahti, E., Engvall, E., and Hayman, E. G. (1981). *Collagen Res.* **1,** 95–128.

Ruoslahti, E. and Pierschbacher, M. D. (1987). *Science* **238,** 491–497.

Ruoslahti, E. and Engvall, E. (1980). *Biochem. Biophys. Acta* **631,** 350–358.

Sadoul, J., Peyron, J.-F., Balloti, R., Debant, A., Fehlmann, M., and Van Obberghen, E. (1985). *Biochem. J.* **227,** 887–892.

Santamaria L., Martinez-Onsurbe, P., Paniagua, R., and Nistal, M. (1990). *Intl. J. Androl.* **13,** 135–146.

Skinner, M. K., Stallard, B., Anthony, C. T., and Griswold, M. D. (1989). *Mol. Cell. Endocrinol.* **66,** 45–52.

Skinner, M. K., Tung, P. S., and Fritz, I. B. (1985). *J. Cell Biol.* **100,** 1941–1947.

Terracio, L., Gullberg, D., Rubin, K., Craig, S., and Borg, T. K. (1989). *Anat. Res.* **223,** 62–72.

Terracio, L., Rubin, K., Balog, E., Jyring, R., Gullberg, D., Carver, W., and Borg, T. K. (1991). *Circ. Res.* **68,** 734–743.

Terranova, V. P., Aumailley, M., Sultan, L. H., Martin, G. R., and Kleinman, H. K. (1986). *J. Cell Physiol.* **127,** 473–479.

Tung, P. S., Skinner, M. K., and Fritz, I. B. (1984). *Biol. Reprod.* **30,** 199–211.

Vernon, R. B., Lane, T. F., Angello, J. C., and Sage, H. (1991). *Biol. Reprod.* **44,** 157–170.

Vuori, K. and Ruoslahti, K. (1993). *J. Biol. Chem.* **268,** 21,459–21,462.

Wu, N. and Murono, E. P. (1994). *Mol. Cell. Endocrinol.* **106,** 99–109.

Yamada, K. M. K., Olden, K., and Hahn, L.-H. E. (1980). In: *The Cell Surface: Mediator of Developmental Processes.* Subtelny S. and Wessels, N. K. (eds.). Academic, New York, pp. 43–77.