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Modulation of Mouse Preimplantation Embryo Development by Acrogranin (Epithelin/Granulin Precursor)

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Preimplantation mammalian embryos in culture secrete autocrine growth factors into the surrounding medium that, in turn, stimulate the development of the embryos. The full complement of these factors is unknown. Since one hallmark of embryo development is the formation of an epithelium, the trophectoderm, we tested the hypothesis that one such embryo-derived growth factor is acrogranin (epithelin/granulin precursor), a factor that possesses growth-regulatory activities principally toward epithelial cells. We found that acrogranin mRNA was expressed in preimplantation mouse embryos with the transcript levels rising to their highest point in blastocysts, coincident with the appearance of the trophectoderm. Indirect immunofluorescence confocal microscopy of preimplantation mouse embryos at different developmental stages revealed that acrogranin immunostaining was most concentrated in the trophectoderm of blastocysts. Immunoblotting and immunoprecipitation experiments demonstrated that the embryos secreted acrogranin into the surrounding medium. To determine how altering the levels of acrogranin in the culture medium surrounding the embryos might affect embryonic growth and development, acrogranin protein levels in the culture medium were decreased with a function-blocking antibody or increased by adding the purified acrogranin to the medium. In both a concentration-dependent and a reversible manner, affinity-purified anti-acrogranin antibody significantly inhibited the development of eight-cell embryos to the blastocyst stage compared to controls (no added immunoglobulin or nonspecific IgG). Furthermore, embryo cell numbers were significantly decreased in the presence of the highest concentrations of acrogranin antibody compared to control embryos. Exogenous acrogranin added to cultures of eight-cell embryos accelerated the time for the onset of cavitation, as well as stimulating the rate of blastocoel expansion and increasing the number of trophectoderm cells compared to controls. These results indicate that acrogranin can regulate the appearance of the epithelium in the developing mouse blastocyst, the growth of the trophectoderm, and/or the function of the embryonic epithelium.

INTRODUCTION

The blastocyst consists of two cell types: the inner cell mass (ICM) and the trophectoderm. The ICM is the precursor of the embryo proper while the trophectoderm, a fluid-transporting epithelial cell layer encasing the embryo, is the first differentiated embryonic cell type and subsequently gives rise to the placenta. Blastocyst formation entails cell division, differentiation, and apoptosis that occur in a coordinated fashion (Fleming and Johnson, 1988). The preimplantation embryo expresses a variety of peptide growth factors that likely regulate each of these processes (Kaye, 1997). For example, TGF-α can both stimulate the rate of

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blastocoel expansion and serve as a cell survival factor for ICM cells (Brison and Schultz, 1998; Dardik and Schultz, 1991a). Little is known, however, regarding the role of peptide growth factors in epithelial development in the preimplantation embryo.

A potential candidate for such a function is acrogranin, a factor that regulates epithelial cell growth and is highly expressed in tissues of the reproductive tract (Baba *et al.*, 1993; Shoyab *et al.*, 1990). One of our laboratories originally isolated and characterized acrogranin as a 67,000 M_r glycoprotein (Anakwe and Gerton, 1990). The sequence of guinea pig and mouse testis cDNAs encoding acrogranin demonstrated that this protein is synonymous with the precursor of the epithelin and granulin peptides (Baba *et al.*, 1993; Bateman *et al.*, 1990; Shoyab *et al.*, 1990). Acrogranin mRNA is ubiquitously expressed in a variety of human, mouse, and guinea pig tissues (Baba *et al.*, 1993; Bhandari *et al.*, 1993; Plowman *et al.*, 1992). Interestingly, acrogranin mRNA and protein are most highly expressed in the testis, kidney, epididymis, vas deferens, seminal vesicles, ovary, uterus, and lung, suggesting that acrogranin plays a role in reproduction.

Acrogranin and its derivatives (epithelins, granulins, transforming growth factor e, and others) are regulators of cell growth. Epithelins 1 and 2 (~6000 M_r) were originally purified from rat kidney extracts (Shoyab *et al.*, 1990). Epithelin 1 stimulates the proliferation of murine keratinocytes in culture, whereas epithelin 2 inhibits the action of epithelin 1. In contrast, both epithelin 1 and epithelin 2 inhibit the growth of the epidermoid carcinoma A431 cells and the proliferation of the human breast carcinoma cell line MDA-MB-468 (Culouscou *et al.*, 1993; Shoyab *et al.*, 1990). The granulins isolated from human leukocytes and rat bone marrow are structurally related to epithelins and are derived from the same precursor, acrogranin (Bateman *et al.*, 1990). Granulins have also been identified in fish (Belcourt *et al.*, 1993, 1995) and recently have been isolated from human urine (Sparro *et al.*, 1997). A larger fragment of acrogranin (23,000–25,000 M_r), termed transforming growth factor e (for epithelium), is an autostimulatory transforming growth factor required for the optimal growth of SW-13 adrenal carcinoma cells in soft agar (Halper and Moses, 1987; Parnell *et al.*, 1992). More recently, the full-length acrogranin protein has been demonstrated to be an autocrine growth regulator for a highly tumorigenic cell line (Zhou *et al.*, 1993) and for a 3T3 cell line null for the type 1 insulin-like growth factor receptor (Xu *et al.*, 1998).

As a consequence of the high expression of acrogranin in reproductive tissues, as well as recognizing that the first sign of cellular differentiation in the preimplantation mammalian embryo is the formation of the initial epithelium, the trophectoderm, we tested the hypothesis that acrogranin regulates blastocyst formation with the concomitant formation of the trophectoderm. We report that (1) acrogranin mRNA and protein are expressed in the preimplantation mouse embryo (with the levels rising to highest amount at the blastocyst stage), (2) acrogranin is secreted into the culture medium by blastocysts, (3) a function inhibiting anti-acrogranin antibody reversibly inhibits development of eight-cell embryos to the blastocyst stage, and (4) addition of acrogranin to the culture medium stimulates blastocyst formation.

MATERIALS AND METHODS

Oocyte and Embryo Collection and Culture

Female mice (CF-1, 6 weeks of age; Harlan Industries, Indianapolis, IN) were injected with 5 IU of pregnant mare serum gonadotropin (PMSG) and, 48 h later, with 5 IU of human chorionic gonadotropin. The medium for collection of oocytes and embryos was bicarbonate-free minimal essential medium (Earl's salts) supplemented with polyvinylpyrrolidone (3 mg/ml), pyruvate (100 μg/ml), gentamicin (10 μg/ml), and 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes), pH 7.2 (MEM/PVP) (Manejwala *et al.*, 1986). Fully grown mouse oocytes were obtained from PMSG-primed females and freed of attached cumulus cells; to inhibit the resumption of meiosis, 0.2 mM 3-isobutyl-1-methylxanthine was present in the collection medium. For embryo collection, females were paired with B6D2F1/J males (The Jackson Laboratory, Bar Harbor, ME). At appropriate times following mating, one-cell, two-cell, four-cell, eight-cell, morula, and blastocyst stage embryos were flushed from the excised reproductive tracts into bicarbonate-free MEM/PVP. The embryos were cultured at 37°C in 50 μl of KSOM medium (Erbach *et al.*, 1994) under mineral oil in a humidified atmosphere of 5% CO_2 in air.

Oocyte and Embryo RNA Preparation and Semiquantitative RT-PCR Assay

The preparation of RNA from oocytes and embryos and reverse transcription were performed as described previously (Temeles *et al.*, 1994). Prior to the isolation of RNA, 0.125 pg of rabbit globin mRNA (BRL) was added per embryo; this RNA serves as a standard to normalize the differences in RNA recovery and efficiency of the RT-PCR procedures. Oligo(dT)-primed reverse transcription was conducted on 100 oocyte and embryo equivalents. The polymerase chain reactions were performed in 100 μl of 10 mM Tris–HCl, pH 8.3, containing 50 mM KCl, 1.45 mM $MgCl_2$, the four dNTPs at 0.2 mM each, 5 μCi [α-^{32}P]dCTP (sp act 3000 Ci/mmol; Amersham), 2.5 units AmpliTaq polymerase, 20 pmol (0.4 μM) each of appropriate 3′ and 5′ primers, and a volume of the reverse transcription reaction. For acrogranin, the forward primer was CGAAAGAAGATTCCTCGCTGGGAC, corresponding to nucleotides 1732–1755 of pMG1, and the reverse primer was AGAACACCTGTGGCTCGGGAAGAG, corresponding to nucleotides 2039–2062 of pMG1 (Baba *et al.*, 1993). The basic PCR program was a 95°C soak for 1 min, followed by a cycle program of 95°C for 10 s, then 60°C for 15 s. Following PCR, the tubes were chilled briefly on ice and centrifuged to collect any condensation. Then 25-μl aliquots were removed and treated with 2 μl of a 20 μg/ml solution of RNase for 10 min at room temperature to digest any remaining carrier RNA. After the addition of 5 μl of 6× loading buffer (0.25% bromophenol blue in 40% sucrose), 13-μl aliquots of each sample were subjected to electrophoresis on a 4% agarose gel (Manejwala *et al.*, 1991). The bands were located under UV light and excised with a razor blade. The incorporation of radiolabeled deoxycytidine triphosphate (dCTP) in each gel slice was determined by Cerenkov counting.

Immunofluorescence and Laser-Scanning Confocal Microscopy

Oocytes and embryos were collected and the zonae pellucidae were removed with acid Tyrode's solution (Bornslaeger and Schultz, 1985). The zona pellucida-free oocytes or embryos were analyzed by indirect immunofluorescence and laser-scanning confocal microscopy as described previously (Stein *et al.*, 1997). The primary antibody was rabbit anti-acrogranin (Anakwe and Gerton, 1990). Controls for indirect immunofluorescence experiments included the use of preimmune serum as the primary serum and the omission of the primary serum (secondary antibody alone).

Metabolic Labeling of Mouse Embryos and Spermatogenic Cells with [^{35}S]Cysteine and Immunoprecipitation of Acrogranin

Mouse embryos. Early cavitating blastocysts (500) were collected and incubated in the presence of 50 μCi [^{35}S]cysteine (sp act ~1075 Ci/mmol; New England Nuclear (NEN), Boston, MA) in a 50-μl drop of KSOM medium without albumin or added amino acids. Cysteine was used as the radiolabeled precursor because approximately 15% of the amino acids in acrogranin are cysteine (Baba *et al.*, 1993). The embryos were then incubated for 12 h at 37°C under a 95% air:5% CO_2 atmosphere. The resulting "conditioned medium" containing secreted ^{35}S-radiolabeled proteins was recovered by aspiration. Embryos and conditioned medium were stored at −70°C prior to being subjected to immunoprecipitation.

Spermatogenic cells. A mixed population of guinea pig pachytene spermatocytes, round spermatids, and condensing spermatids was suspended in cysteine-free germ cell minimal essential medium consisting of Eagle's minimal essential medium with Earle's salts supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, 6 mM lactate, 1 mM pyruvate, and 15 mM Hepes (Gerton and Millette, 1984). A suspension of 8×10^6 cells in 5 ml was added to 60-mm plastic dishes. [^{35}S]Cysteine (sp act ~1075 Ci/mmol; NEN) was added to a final concentration of 50 μCi/ml. The cells were incubated for 12 h in a humidified incubator at 33°C and 95% air:5% CO_2.

Immunoprecipitation. After the embryos and cells were lysed with PBS containing 1% sodium cholate, 0.1% SDS, 0.02% sodium azide, 40 μg/ml aprotinin, 40 μg/ml leupeptin, and 4 mM *p*-aminobenzamidine, PMSF dissolved in ethanol was added to a final concentration of 10 mM. Immunoprecipitations were then performed using rabbit anti-acrogranin as the primary antibody (Anakwe and Gerton, 1990). The immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the procedure of Laemmli (1970). After SDS–PAGE, the gels were fixed in 50% methanol and dried. Radioactivity was detected by phosphorimaging using the Storm System (Molecular Dynamics, Sunnyvale, CA). Immunoblotting was carried out as previously outlined with antibody to acrogranin (Anakwe and Gerton, 1990) using enhanced chemifluorescence and detection with the Storm System.

Acrogranin Purification

Acid extraction. The purification of acrogranin was performed using a modification of the procedure described by Anakwe and Gerton (1990). Guinea pig testes were separated from the tunicae albugineae and homogenized for 30 s in an extraction buffer made up of 1 mM HCl (pH 3.0) plus 5 mM benzamidine. The ratio of the testes to the buffer was 1:2 (w/v). The homogenate was stirred for 1 h at 4°C. Following centrifugation at 10,000*g* for 30 min, the supernatant was collected and adjusted to pH 6.0 with 1 N NaOH. The precipitate formed was removed by centrifugation. The supernatant was adjusted to pH 3.0 with 1 M HCl and dialyzed against several changes of extraction buffer for over 35 h. The sample was frozen in aliquots and stored at −70°C until used.

Sephacryl S-200 column chromatography. The acid extract of testes (180 mg of protein in 50 ml) was loaded on a Sephacryl -S-200 column (4.8 × 60 cm) equilibrated in 0.05 M sodium acetate and 0.2 M NaCl, pH 3.5. Proteins were eluted in 18-ml fractions at a flow rate of 30 ml/h while absorbance was monitored at 280 nm. Fractions containing acrogranin (as detected by immunoblotting) were pooled.

Affinity chromatography on a concanavalin A–Sepharose column. To the pooled material from the Sephacryl S-200 column, *m*-aminobenzamidine was added to a final concentration of 0.5 mM. The pH was adjusted to 7.4 and the sample was applied to a concanavalin A–Sepharose column (2.5 × 13 cm) that had been equilibrated with 0.5 M NaCl, 0.02 M Tris–HCl, pH 7.4, and 0.5 mM *m*-aminobenzamidine. The concanavalin A-binding proteins were eluted with 0.5 M of α-methyl-D-mannopyranoside dissolved in the same buffer. The eluate was immediately concentrated in an Amicon ultrafiltration cell fitted with a YM30 membrane and then dried in a Jouan Concentrator System (Jouan, Inc., Winchester, VA).

Ion-exchange chromatography. The pooled material from the preceding step was diluted in 5 ml of 0.02 M imidazole, pH 7.0, and run on a Millipore ConSep LC 100 System, using a HiTrap-Q 5 × 5-ml column (Pharmacia Biotech). The column was eluted with a gradient 0 to 1 M NaCl in 0.02 M imidazole, pH 7.0. Fractions exhibiting only acrogranin were pooled, dialyzed to remove salt, and lyophilized prior to resuspension and testing on embryos. A single band of 67,000 M_r was detected in the final product.

Embryo Collection and Culture in the Presence of Antibody or Acrogranin

Eight-cell embryos were collected about 68 h post-hCG from superovulated CF-1 female mice mated to B6D2F1/J males as described above. The embryos were placed in 20-μl drops of KSOM medium containing essential and nonessential amino acids (Gibco BRL, Grand Island, NY) (Ho *et al.*, 1995) and incubated under mineral oil (Sigma) at 37°C in a humidified atmosphere containing 5% CO_2. Four different concentrations of affinity-purified anti-acrogranin antibody (0.03, 0.15, 0.30, and 0.60 μM) were tested. Medium alone and nonspecific rabbit IgG (0.60 μM) were used as controls. Each experimental group consisted of 20 embryos in 20-μl drops. The embryos were incubated under oil for 47 h at 37°C in an atmosphere containing 5% CO_2. They were then scored for the onset of cavitation at 88 and 115 h post-hCG. In other culture groups, the embryos were removed from the antibody after 14 h, washed through several drops of medium, and then cultured in fresh drops of antibody-free medium for another 33 h. These embryos were scored for the incidence of cavitation at 82, 88, 96, and 115 h post-hCG.

Three different concentrations of purified acrogranin (0.01, 0.02, and 0.05 μM) were tested for effects on embryo growth and development. Medium alone was used as a control. Each experimental group consisted of 20 embryos in 20-μl drops of KSOM medium containing essential and nonessential amino acids (Ho *et al.*, 1995). The embryos were incubated under oil at 37°C in an atmosphere containing 5% CO_2. Starting at 45 h, the embryos were

scored for the onset of cavitation every 4 h until 96 h and, finally, at 110 h post-hCG.

Differential Labeling of the Inner Cell Mass and Trophectoderm

The nuclei of blastocyst trophectoderm and ICM cells were differentially labeled with polynucleotide-specific fluorochromes following a modification of the "immunodissection" procedure of Handyside and Hunter (1984). Briefly, the zonae pellucidae were removed in acid Tyrode's solution and zona-free blastocysts were washed in medium M2 containing 5% BSA. The embryos were then incubated with 10% anti-mouse T-cell antiserum (Thy–1; Accurate Chemical and Scientific Corp., Westbury, NY) for 10 min at 37°C. After washing in M2, blastocysts were incubated in a 1:10 dilution of guinea pig complement (Sigma) in M2 containing 40 μg/ml bisbenzimide (Hoechst 33258) and 20 μg/ml propidium iodide. Labeled blastocysts were washed several times in M2 plus 1% BSA. Embryos with labeled nuclei were individually mounted on microscope slides. ICM nuclei that were labeled with bisbenzimide appeared blue and trophectoderm nuclei that were labeled with the combination of propidium iodide and bisbenzimide appeared pink. After photography and recording of the staining pattern, the embryos were carefully squashed and disaggregated by gently pressing the coverslip with a pencil eraser; the slide was then examined again for counting.

Measurement of Blastocoel Expansion

The rate of increase in the volume of the blastocoel was measured as described previously (Manejwala *et al.*, 1986). The nascent cavities of the embryos were measured and then the embryos were placed in individual drops of medium alone or medium containing acrogranin. The axes of the blastocoels of individual embryos were measured as a function of time on an inverted microscope at 200-fold magnification; the focal plane was adjusted to measure the long and short axes of the cavity, and the volume of the blastocoel was calculated assuming a prolate spheroid shape ($V = (4/3)\pi ab^2$). For some experiments the initial volume of a blastocoel was subtracted from the size of the cavity at a later time point to yield the relative change in blastocoel volume.

Statistical Analysis

One-way analysis of variance was employed to determine differences between groups. When differences existed, the *t* test was used to determine which means were significantly different. A value of $P < 0.05$ was considered statistically significant. The JMP version 3 statistical package was used for all analyses.

RESULTS

Expression of Acrogranin mRNA from the Embryonic Genome during Preimplantation Embryo Development Precedes the Formation of the Trophectoderm

The expression of the acrogranin mRNA in the preimplantation mouse embryo was examined using a semiquantitative RT-PCR assay (Fig. 1). By arbitrarily setting the signal from blastocyst as 100 units, we found that the relative level of acrogranin mRNA started at about 80–85 units in oocytes, dropped below 75 units in eggs, continued to decline to ~60 units in one-cell zygotes, and then dropped precipitously to the ~7 unit level in two-cell embryos. The signal then returned to the ~80 unit level by the eight-cell stage, which precedes the morula stage and subsequent blastocyst stage (100 unit level) when the epithelium is first formed. This pattern of expression is typical of many genes that are expressed in the preimplantation embryo and reflects the replacement of maternal mRNAs with embryonically expressed transcripts (Schultz, 1993).

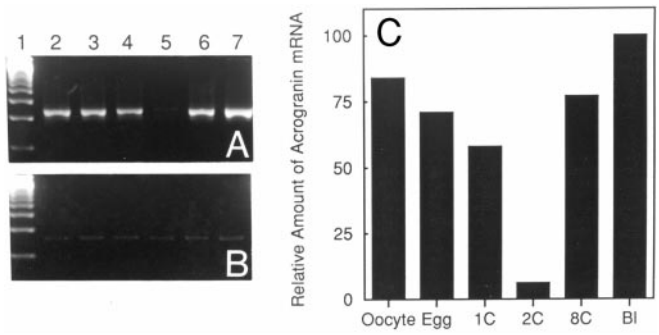

FIG. 1. Expression of acrogranin in the preimplantation mouse embryo as determined by RT-PCR. Left: Ethidium bromide-stained gel. (A) Amplicons from acrogranin primers. (B) Amplicons from α-globin primers (exogenously added control). Right: Ratio of ^{32}P-labeled amplicons. (C) Using the signal from blastocyst as 100%, the relative level of acrogranin started at about 80–85% in oocytes, dropped below 75% in eggs, continued to decline to ~60% in one-cell zygotes, and then dropped to ~7% in two-cell embryos. At the eight-cell stage the signal returned to the 80% level and finally to the 100% level at the blastocyst stage. This experiment was performed twice with similar results. The values from one experiment are shown.

Acrogranin Protein Expression during Preimplantation Embryo Development Suggests an Apical Secretion Pattern

Acrogranin expression and localization in preimplantation embryos at different developmental stages were examined by indirect immunofluorescence using the anti-acrogranin antibody (Fig. 2). Acrogranin immunostaining was detected in oocytes and appeared progressively fainter and disappeared in the subsequent stages two, four, and eight cell). The immunofluorescence signal then reappeared in morula stage and became much stronger in the apical surface of the trophectoderm and, to a lesser extent, the inner cell mass of blastocysts (Fig. 2). The expression patterns in the various embryo stages correlated with the RT-PCR data. Neither control (preimmune serum as the primary serum or secondary antibody alone) resulted in any appreciable staining of the embryos (data not shown).

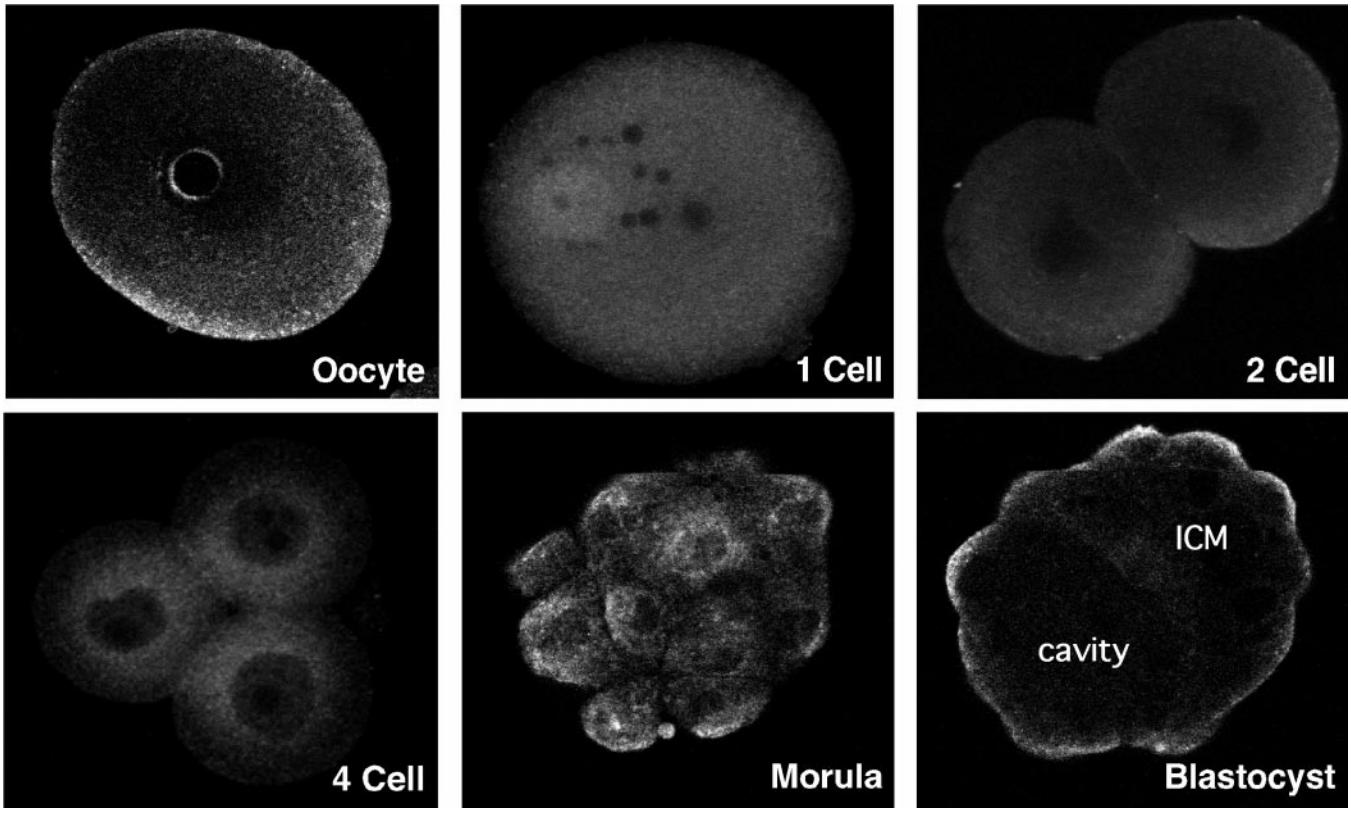

FIG. 2. Localization of acrogranin in the preimplantation mouse embryos. Acrogranin immunostaining was detected in oocytes, appeared progressively fainter, and disappeared in the subsequent embryos stages (one, two, and four cell), but then reappeared in the morula stage and became much stronger in the apical surface of the trophectoderm. Although not apparent in this micrograph, we occasionally detected staining in the inner cell mass of the blastocyst. ICM, inner cell mass; cavity, blastocoelic cavity. The immunofluorescence experiments were performed twice with identical results.

Mouse Blastocysts Secrete Acrogranin into the Culture Medium

The immunofluorescence staining in the outer periphery of the trophectoderm suggested that acrogranin might be secreted into the medium surrounding the embryos. To test this possibility, conditioned medium was collected from blastocysts after a 12-h culture period. The secreted proteins were separated by SDS–PAGE and analyzed by immunoblotting using the anti-acrogranin antibody. An immunoreactive band with an M_r 67,000 was detected in the culture medium (Fig. 3A); the size of the polypeptide was consistent with our previous results with guinea pig spermatogenic cells (Anakwe and Gerton, 1990).

To verify that blastocysts were capable of synthesizing and secreting acrogranin into the medium, blastocysts were cultured in the presence of [^{35}S]cysteine and the metabolically radiolabeled, conditioned medium was collected after 12 h. The radiolabeled secreted proteins were used for the immunoprecipitation of acrogranin. The antibody specifically immunoprecipitated acrogranin as a single band of protein with M_r 67,000 from conditioned medium and blastocysts, as well as from the guinea pig spermatogenic cells that were used as positive controls (Fig. 3B). Preimmune antiserum failed to immunoprecipitate any radiolabeled proteins from blastocyst-conditioned medium or the guinea pig spermatogenic cell extract, demonstrating the specificity of the protocol. Although background bands were seen with preimmune serum in the precipitates of blastocysts, no band comigrated with the authentic acrogranin. These results indicate that the embryos synthesized acrogranin and secreted it into the extracellular milieu surrounding the blastocysts.

Anti-acrogranin Inhibits Blastocyst Formation

The previous set of experiments demonstrated that the embryos secrete acrogranin into the culture medium. To test whether the acrogranin could act as autocrine regulator of the embryo growth and/or differentiation, the effect of the function-blocking anti-acrogranin antibody on embryo development was determined. Compacted eight-cell mouse embryos were cultured in the presence in four different concentrations of affinity-purified anti-acrogranin antibody

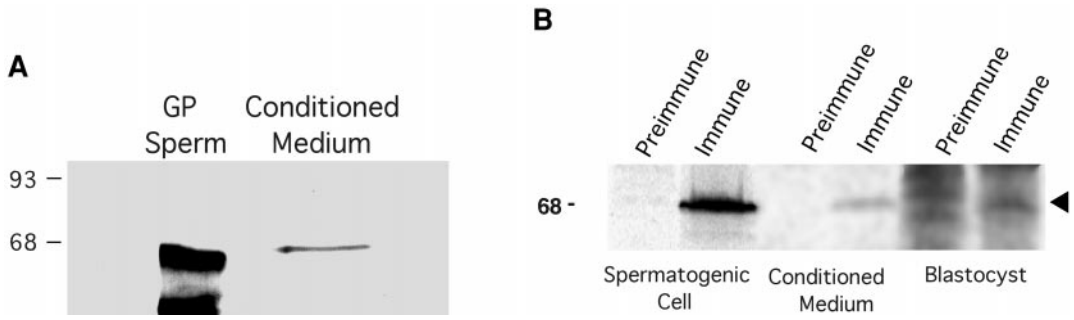

FIG. 3. Detection of secreted acrogranin in blastocyst-conditioned medium. (A) Immunoblot analysis of acrogranin secretion as a single band by mouse blastocysts into the culture after 12 h in culture. An extract of guinea pig (GP) sperm was used as a positive control. (B) Immunoprecipitation of acrogranin. Mouse blastocysts were metabolically labeled overnight with [^{35}S]cysteine (50 μCi/ml). A single band of acrogranin at 67,000 M_r (triangle) was immunoprecipitated from blastocysts, embryo-conditioned medium, and guinea pig spermatogenic cells used as a positive control. This experiment was performed twice with similar results. Numbers to the left refer to molecular weights ($\times 10^3$) of standard proteins.

(0.03, 0.15, 0.30, and 0.60 μM), medium alone, or nonspecific IgG for 47 h (Fig. 4) and scored for the onset of cavitation at 88 and 115 h post-hCG. Subsequently, the number of cells per embryo was determined. In other groups of embryos, the embryos were cultured for 14 h, washed, and transferred to antibody-free medium. After being cultured for another 33 h, the embryos were then scored for the incidence of cavitation at 82, 88, 96, and 115 h post-hCG (Fig. 5).

In six experiments, we consistently observed that affinity-purified anti-acrogranin antibody significantly ($P <$ 0.05) inhibited the development of compacted, eight-cell embryos to the blastocyst stage in a dose-dependent manner (Figs. 4 and 6). Controls (no added IgG or nonspecific rabbit IgG) were unaffected. After 20 and 47 h of culture, the percentages of embryos that cavitated at 88 and 115 h post-hCG were greatly decreased in those groups of embryos treated with the high concentrations of anti-acrogranin antibody compared to controls (no added IgG and nonspecific rabbit IgG) (Fig. 4). This inhibitory effect was reversible; when the antibody was removed by washing, the treated embryos (with the four doses) progressively

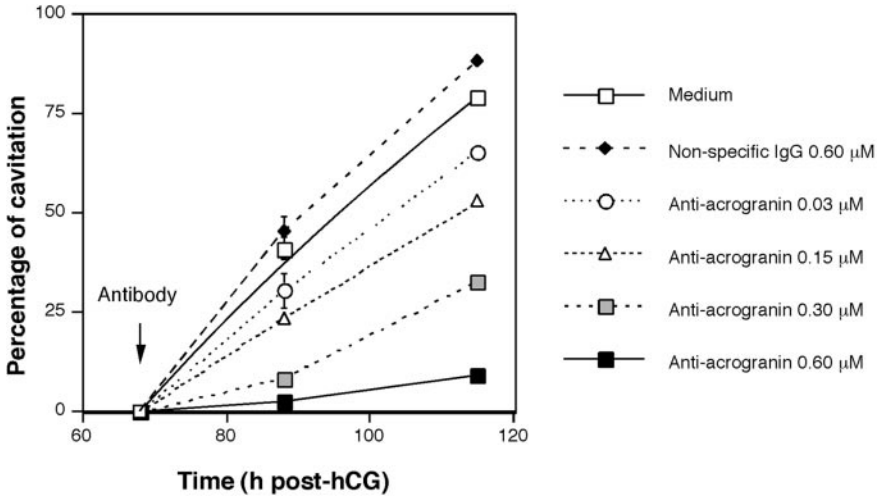

FIG. 4. Inhibition of cavitation by anti-acrogranin. Mouse embryos were cultured from the eight-cell stage through the blastocyst stage in medium alone, with nonspecific IgG, and with four different doses of affinity-purified anti-acrogranin antibody. The embryos were cultured for 47 h and scored for cavitation at 88 and 115 h post-hCG. The three high concentrations of acrogranin antibody (0.15, 0.030, and 0.60 μM) inhibited the development of the embryos in significant form ($P < 0.05$) compared with the controls (medium alone and nonspecific IgG). The data are expressed as means ± SEM from six experiments. In this and subsequent graphs, error bars represent the SEM; if no error bars are shown for a given point, the SEM was smaller than the size of the symbol.

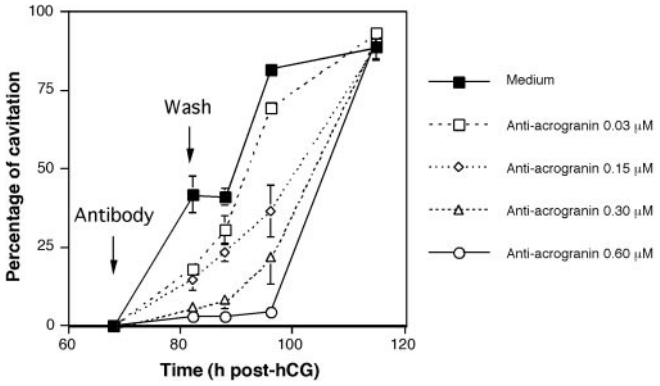

FIG. 5. Reversibility of the effect of anti-acrogranin antibody on cavitation. Mouse embryos were cultured from the compacted eight-cell stage through the blastocyst stage in medium alone, with nonspecific IgG, and with four different doses of affinity-purified anti-acrogranin antibody. Following 14 h of culture, the extent of cavitation at 88 h post-hCG was greatly decreased in embryos treated with anti-acrogranin antibody (0.15, 0.30, and 0.60 μM) compared with the controls (medium and nonspecific IgG). When the antibody was removed by washing, the treated embryos cavitated and there were no significant differences in the percentage of cavitating embryos between the groups. The data are expressed as means $\pm$ SEM from four experiments with 15–20 embryos per data point for each experiment.

cavitated, such that by 115 h post-hCG there were no significant differences in the percentage of cavitating embryos compared to the controls (Fig. 5). We also noted that the embryos cultured in medium alone exhibited a pause in the development of cavities following the washing. This delay is consistent with the removal of endogenously secreted acrogranin from the embryo-conditioned medium during this step. Cavitation presumably resumed once the acrogranin (and/or other growth factors) secreted by the embryos reached a threshold level.

The antibody-treated embryos were able to escape from the zonae pellucidae but hatching was delayed by approximately 14 h compared to the control (medium alone) (Fig. 7). The reversible nature of the antibody minimizes the likelihood that the antibody has a direct toxic effect on the embryos. Consistent with this conclusion is the finding that vital dye staining of the embryos cultured in the presence of the anti-acrogranin antibody did not detect an increase in the incidence of dead or dying cells (data not shown).

The embryos that did not cavitate were clearly smaller than those cultured in medium alone or nonspecific IgG (Fig. 6). The smaller sizes could simply result from a reduced ability of the treated embryos to pump fluid into or maintain fluid within nascent blastocoelic cavities. Another possibility was that the treated embryos consisted of fewer cells. To examine this question, we recovered eight-cell embryos at 67 h post-hCG and cultured these for 48 h in the presence of anti-acrogranin antibody. The number of cells per embryo was determined after staining the nuclei with bisbenzimide. Under these conditions, blastocysts develop quite readily in the control group (medium or nonspecific IgG treated). Interestingly, there was a dose-

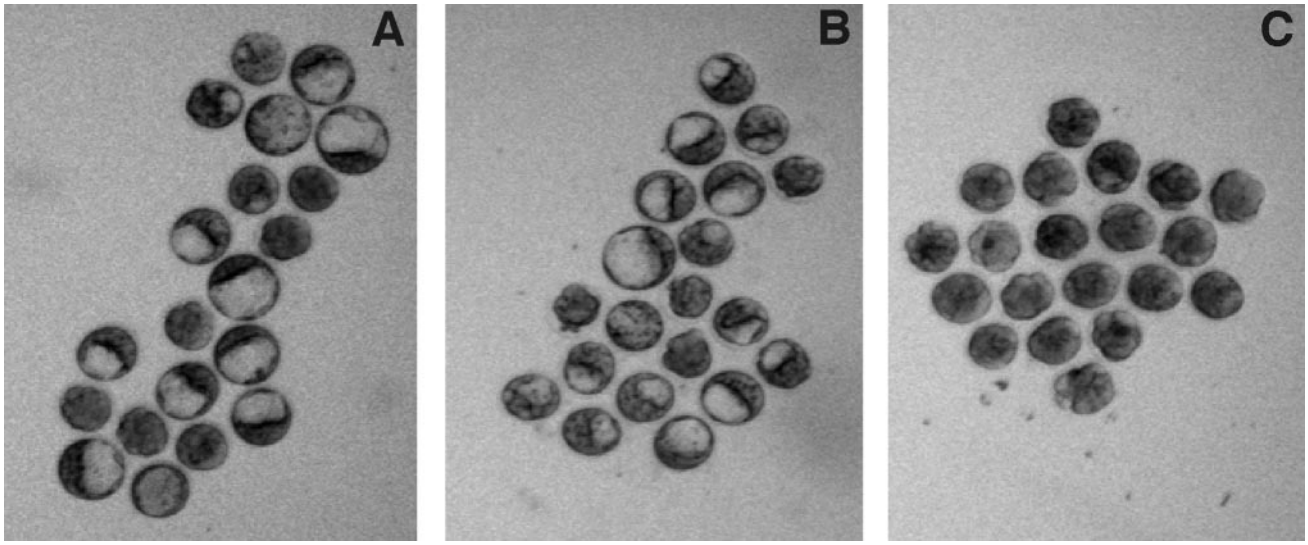

FIG. 6. Morphology of embryos after treatment with anti-acrogranin. Preimplantation mouse embryos after 28 h in culture (96 h post-hCG) with (A) medium alone, (B) medium with nonspecific IgG (0.60 μM), and (C) medium with anti-acrogranin antibody (0.60 μM). The morphology of the nonspecific IgG-treated embryos did not differ from that of the control embryos although the anti-acrogranin treated embryos displayed minimal cavitation. The experiment was performed four times; representative images are shown.

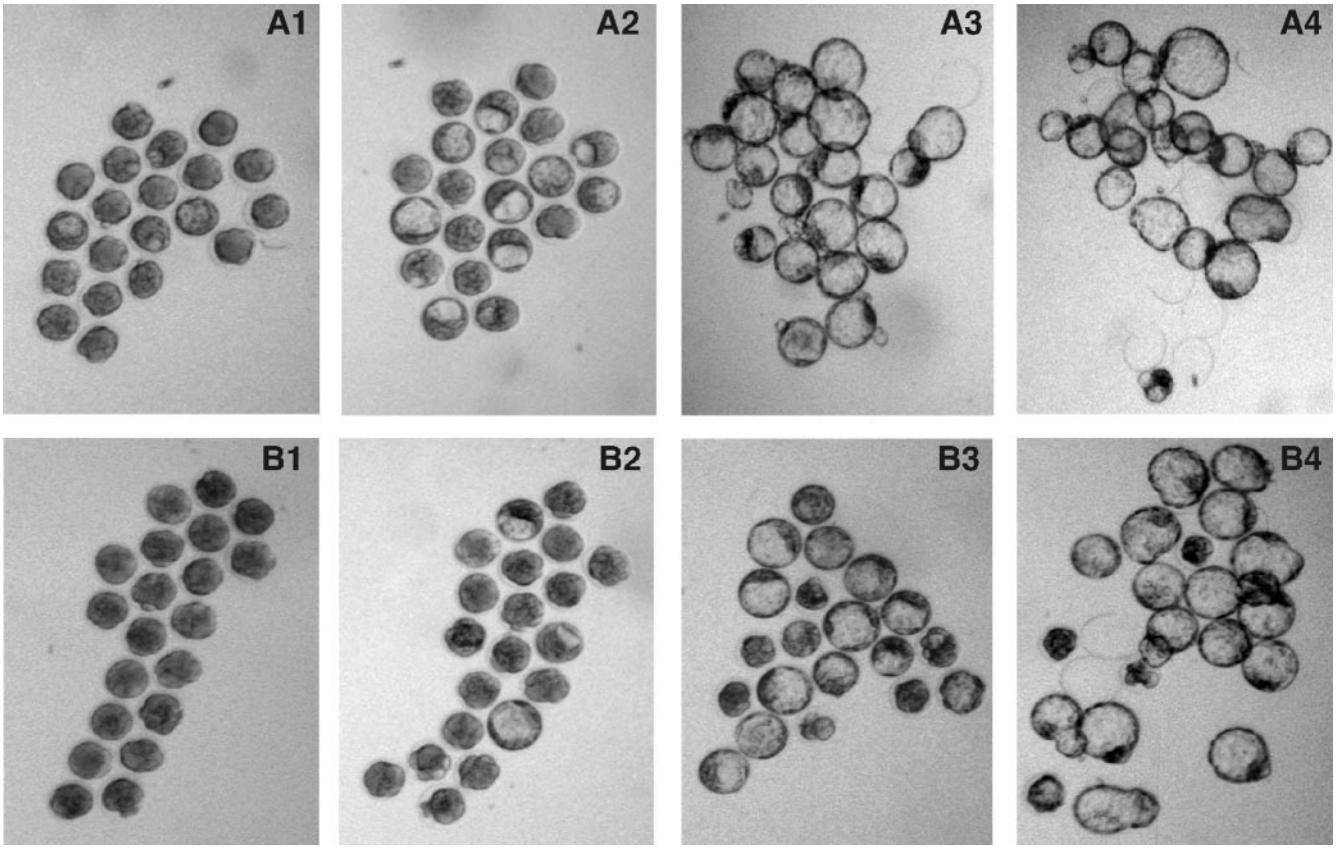

FIG. 7. Morphology of anti-acrogranin-treated embryos after removal of the antibody and subsequent culture. Preimplantation mouse embryos cultured in medium alone (A) and treated with affinity-purified anti-acrogranin 0.60 μM (B). At 14 h in culture, the antibody was removed by washing (A1, B1). At 6 h after the anti-acrogranin was removed, treated embryos began cavitation (B2) but the extent of blastocoel formation was less than that found in control embryos (A2). At 24 h after the antibody was removed, the treatment group consisted of healthy blastocysts (B3) but no hatching from the zonae pellucidae was apparent; control embryos (A3) were hatching at this time. However, by 48 h following the removal of anti-acrogranin, the treated embryos had hatched (B4). The experiment was performed four times; representative images are shown.

dependent and statistically significant ($P < 0.05$) lower number of cells per embryo cultured in the presence of increasing concentrations of anti-acrogranin antibody (Fig. 8). These results indicate that the acrogranin secreted by the embryos acted as an autocrine regulator of cell differentiation (blastocoel formation) and cell proliferation.

Purified Acrogranin Stimulates Blastocoelic Cavity Formation and Expansion

Since the anti-acrogranin antibody inhibited blastocyst formation, we hypothesized that the addition of purified acrogranin would stimulate blastocyst formation. To determine the effect of purified acrogranin on preimplantation development, compacted eight-cell mouse embryos were cultured in the presence of three different concentrations of acrogranin (0.01, 0.02, and 0.05 μM), medium alone, or the highest concentration of acrogranin antibody (0.60 μM) for 42 h. Cavitation was scored every 4 h (Fig. 9). Our results show that the highest concentration of acrogranin (0.05 μM) significantly ($P < 0.05$) stimulated embryo development at each time (84 through 96 h) compared to the other groups. At 110 h post-hCG, there were no differences in the percentages of cavitating embryos among groups treated with acrogranin or medium alone. The significant ($P < 0.05$) inhibitory effect was corroborated again with embryos cultured in the presence of anti-acrogranin antibody.

The stimulatory effect of acrogranin on embryo development led us to examine if acrogranin reduced the time of the onset of cavitation, as well as stimulating the rate of blastocoel expansion. The mean times of cavitation, representing the point when visible blastocoels had developed in 50% of the final number of blastocysts, were determined. In eight-cell stage embryos cultured in medium containing the two different doses of acrogranin (0.02 and 0.05 μM), cavitation progressed more rapidly (95.9 $\pm$ 0.10 and 95.0 $\pm$

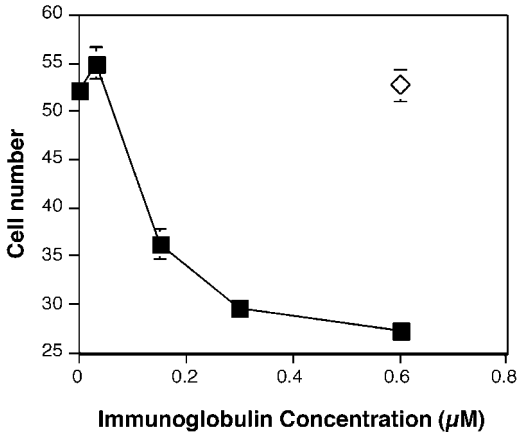

FIG. 8. Inhibition of cell proliferation by anti-acrogranin. Mouse embryos were cultured from the eight-cell stage through the blastocyst stage in medium alone, with nonspecific IgG, and with four different doses of affinity-purified anti-acrogranin antibody. The embryos were cultured for 47 h and cell numbers per embryo counted at 115 h post-hCG. The three high concentrations of acrogranin antibody (0.15, 0.030, and 0.60 μM) significantly blocked the proliferation of blastomeres ($P < 0.05$) compared with the controls (medium alone and nonspecific IgG). Shown are representative data from one of two experiments. For each point on the curve, mean cell numbers per embryo ± SEM were calculated for 16–20 embryos in a treatment group. Affinity-purified anti-acrogranin IgG, closed boxes; nonspecific IgG, open diamond.

0.4 h post hCG, respectively) than in the embryos cultured in medium alone (97.9 ± 0.2 h; Fig. 10). In addition, the increases in blastocoel volume after 110 h post-hCG with either the 0.02 or the 0.05 μM dose of acrogranin (23.11 ± 1.51 or 23.57 ± 2.1 pl, respectively) were statistically significant ($P < 0.05$) compared with medium alone (17.9 ± 0.86 pl) (Fig. 11).

Purified Acrogranin Stimulates Trophectoderm Epithelial Cell Proliferation

To determine whether the factor was affecting the proliferation of cells in the embryos, compacted eight-cell embryos were recovered from mated female mice at 68 h post-hCG. After 24 h in culture (92 h post-hCG), acrogranin was found to stimulate the proliferation of the trophectoderm ($P < 0.05$; Table 1). Embryos treated with 0.02 or 0.05 μM acrogranin had greater numbers of trophectoderm cells compared to embryos cultured in medium alone. There were no statistically significant differences in the numbers of cells in the ICM between the groups (Table 1). As would be expected from the increase in the number of trophectoderm cells, the total cell number increased significantly ($P < 0.05$) in the embryos treated with either dose of acrogranin.

DISCUSSION

In this study we provide the first demonstration that acrogranin expressed by the preimplantation mouse embryo regulates blastocyst formation in an autocrine manner. Full-length acrogranin is synthesized and secreted by the blastocyst. Addition of acrogranin to the culture medium stimulates the incidence of blastocyst formation, reduces the time for the onset of cavitation, stimulates the rate of blastocoel expansion, and increases the number of trophectoderm, but not ICM, cells. Moreover, function-blocking anti-acrogranin antibodies delay the onset of blastocyst formation.

Acrogranin as an Autocrine Regulator of Embryonic Development

Acrogranin joins the list of proteins apically secreted by blastocysts (Dardik and Schultz, 1991b, and references therein). Secretion of acrogranin into the culture medium is consistent with its localization in the apical cortices of the blastomeres; careful examination of the confocal images suggests that the acrogranin is localized within secretory granules near the embryo surface. Immunogold electron microscopy, however, will be required to verify such a localization. Blastocysts also secrete proteins of both trophectoderm and ICM origin into the blastocoel (Dardik and Schultz, 1991b). Although the results presented here suggest a role for embryo-derived acrogranin stimulating trophectoderm differentiation, the embryo-derived and -secreted acrogranin could act as a paracrine regulatory factor on the endometrium, which is also an epithelium. Reciprocally, acrogranin is expressed in the uterus (Baba *et al.*, 1993; Díaz-Cueto and Gerton, unpublished observations) and could function as a uterine-derived factor on embryo development similar to the way that exogenously added acrogranin accelerated blastocyst formation in culture.

Although acrogranin is detected in ICM cells by immunocytochemistry, a pronounced cortical localization facing the blastocoel is not observed, suggesting minimal secretion by ICM cells. Analysis of acrogranin secretion by isolated ICM cells is required to resolve this question. It should be noted that as development of the embryo proceeds, the outer cells of the ICM that face the blastocoel subsequently differentiate into the endoderm, the second epithelium formed during development. It is tempting to speculate that if acrogranin is secreted into the blastocoel by either trophectoderm or ICM cells (or both), the acrogranin could also promote the differentiation of the endoderm since acrogranin appears to modulate the transformation of the outer cells of the embryo into the trophectoderm.

The function of acrogranin in the oocyte is not unknown. Since oocytes secrete proteins that act on the surrounding follicle cells (Eppig, 1991; Lanuza *et al.*, 1998; Vanderhyden *et al.*, 1992), it is possible that oocyte-derived acrogranin could act on the surrounding cumulus cells, which constitute a pseudo-stratified epithelium. Consistent with such a

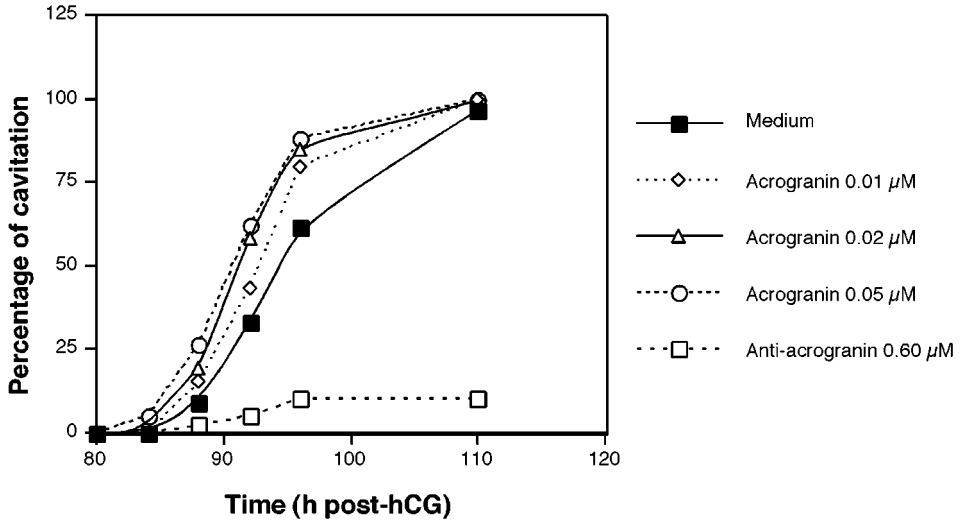

FIG. 9. Effect of acrogranin on blastocoel formation in preimplantation mouse embryos. Mouse embryos were cultured from the compacted eight-cell stage through the blastocyst stage in medium alone, in three different doses of acrogranin, and in one concentration of anti-acrogranin antibody. The embryos were cultured for 42 h and scored for cavitation every 4 h. The highest concentration of acrogranin (0.05 μM) stimulated the development of the embryos in significant form ($P < 0.05$) at each time except at 110 h compared with the control (medium alone). Anti-acrogranin antibody significantly ($P < 0.05$) inhibited blastocoel formation in treated embryos compared with all other groups. The data are expressed as means ± SEM from four experiments with 15–20 embryos per data point for each experiment.

role is our preliminary finding that the ovarian cell type with the highest expression of acrogranin protein is the oocyte (Díaz-Cueto and Gerton, unpublished observations).

Effects of Acrogranin on Differentiation

Acrogranin accelerates the time for the onset of cavitation, as well as stimulating the rate of blastocoel expansion. Acrogranin could affect the expression of components central to these processes. For example, a basolaterally located Na^+/K^+-ATPase is responsible for the vectorial translocation of sodium ions into the blastocoel, and the passive flow of water in response to the osmotic difference generated across the trophectoderm results in expansion of the blastocoel (Biggers *et al.*, 1988). An increase in the expression of the Na^+/K^+-ATPase occurs just prior to the onset of cavitation (Watson *et al.*, 1990a,b). Thus, acrogranin could accelerate the onset of cavitation by up-regulating the expression of the Na^+/K^+-ATPase. In a similar vein, an increase in the Na^+/K^+-ATPase expression or a change in its intrinsic activity could result in an increase in the rate of blastocoel expansion.

Embryos cultured in the presence of anti-acrogranin antibody did not cavitate at the same rate as control embryos. This effect could result from an inhibition of compaction. For example, acrogranin could influence tight junction formation and/or maintenance. The expression of the junction protein ZO-1α^+ appears to be the rate-limiting step in tight junction formation in the blastocyst (Sheth *et al.*, 1997). Stimulation of ZO-1α^+ expression by acrogranin could stimulate the onset of cavitation, as well as the rate of blastocoel expansion, by reducing the paracellular efflux of small ions from the blastocoel. Acrogranin-induced post-translational modifications of tight junction proteins could

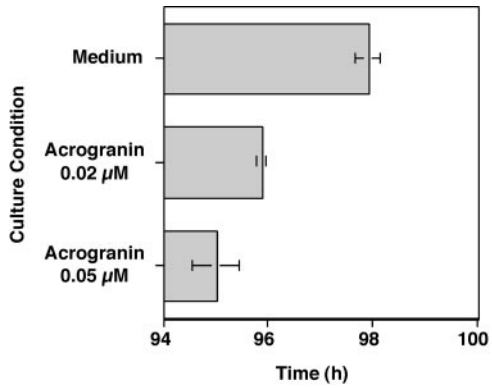

FIG. 10. Exogenously added acrogranin stimulated the onset of cavitation. Mouse embryos were cultured from the compacted eight-cell stage through the blastocyst stage in medium alone or in two doses of acrogranin (0.02 and 0.05 μM). The embryos were cultured for 42 h and scored for cavitation every 4 h. The mean time of cavitation represents the time at which 50% of the final number of blastocysts that cavitated had developed a visible blastocoel. Both doses of acrogranin showed a stimulatory effect on the rate of blastocoel expansion and significantly accelerated the onset of cavitation ($P < 0.05$) compared with the control. The data are expressed as the means ± SEM from five experiments with 15–20 embryos per data point for each experiment.

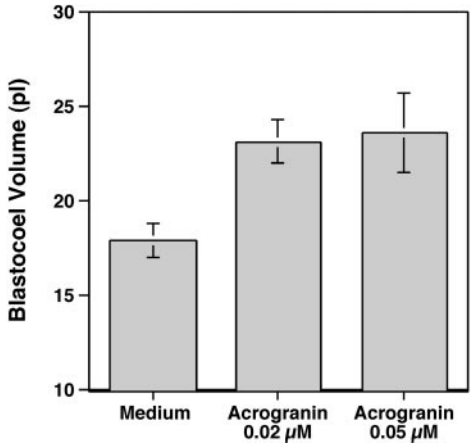

FIG. 11. Acrogranin accelerated the expansion of the blastocoelic cavity in treated embryos. Mouse embryos were cultured from compacted eight-cell stage through the blastocyst stage in medium alone and medium containing two different doses of acrogranin (0.02 and 0.05 μM). After 110 h post-hCG both doses of acrogranin stimulated blastocoel expansion. The difference between the control and both doses of acrogranin was significant ($P < 0.05$). The data are expressed as the means ± SEM from two experiments with 15–20 embryos per data point for each experiment.

also result in an increase in tight junction permeability. For example, an increase in tyrosine phosphorylation is associated with a decrease in tight junction permeability (Mitic and Anderson, 1998).

Acrogranin also selectively stimulates the proliferation of the trophectoderm cells; ICM cell number is unaffected. This contrasts with the selective stimulation of ICM proliferation in response to insulin and IGFs (Harvey and Kaye, 1992a,b), which is likely attributed to their being transcytosed across the trophectoderm, so that they can bind to their cognate receptors that are expressed on the ICM (Heyner *et al.*, 1989; Smith *et al.*, 1993). The ability of acrogranin to stimulate cell proliferation in isolated ICMs is not known. Similar to acrogranin, the cytokine colony-stimulating factor-1 (CSF-1) also accelerates the onset of cavitation and selectively increases the number of trophectoderm cells (Bhatnagar *et al.*, 1995); however, the effect of CSF-1 on the rate of blastocoel expansion was not analyzed in that study. TGF-α/EGF also promotes an increase in the rate of blastocoel expansion. The ability of several different embryo-derived factors to modulate a similar set of autocrine responses constitutes an example of compensatory mechanisms functioning during preimplantation development, and the results presented here add acrogranin to the list of such factors.

Acrogranin Effects on Embryo Differentiation in Relation to Other Studies

Several studies have identified acrogranin or fragments of acrogranin (e.g., epithelins, granulins, TGFe) as factors containing growth-modulatory activity principally toward epithelial cells. In fact, the original identification of the ~6000 M_r epithelins as growth factors, followed by the recognition that the primary structure of acrogranin encompasses the amino acid sequences of all epithelins and granulins, led some to speculate that acrogranin is an inactive precursor for the smaller peptides. Recently, it has become clear that the full-length protein is bioactive and may be more potent than the smaller peptides. For example, the full-length protein is an autocrine growth factor for PC cells and R cells. The PC cell line is a highly tumorigenic, insulin-independent, teratoma-derived cell line isolated from the nontumorigenic, insulin-dependent 1246 cell line (Zhou *et al.*, 1993). Acrogranin is the only purified growth factor known to stimulate the growth of R− cells, mouse embryo fibroblasts null for the type 1 insulin-like growth factor receptor (Xu *et al.*, 1998). Results presented in our studies demonstrate that blastocysts secrete a full-length form of acrogranin and that it is most likely that this form acts directly on the trophectoderm cells of preimplantation mouse embryos in an autocrine manner.

The fact that embryos elicit responses to acrogranin indicates that they possess the necessary machinery to bind the factor and trigger an intracellular signal transduction pathway. The signaling mechanisms elicited in response to acrogranin action are gradually being elucidated. Recently, the epithelin/granulin precursor (i.e., acrogranin) has been shown to activate two mitogenic signaling pathways in the

TABLE 1
Cell Numbers in Blastocysts 24 h after Acrogranin Added to the Culture and 92 h Post-hCG

	Blastocysts examined	Inner cell mass	Trophectoderm	Total cells per blastocyst
Medium	42	15.80 ± 0.67	26.04 ± 1.03[a]	41.71 ± 1.18[b]
Acrogranin (0.02 μM)	31	18.06 ± 0.86	31.51 ± 1.10	49.51 ± 1.52
Acrogranin (0.05 μM)	30	16.60 ± 0.88	30.10 ± 1.19	46.73 ± 1.48

Note. Values represent the means ± SEM of the data derived from the number of blastocysts analyzed in each group.
[a,b] $P < 0.05$ medium vs acrogranin 0.02 and 0.05 μM.

IGF-I receptor-deficient R− cells, the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways (Zanocco-Marani *et al.*, 1999). However, very little is known about the acrogranin receptor. It has been detected by binding assays but the acrogranin receptor appears to be distinct from any other known cytokine or growth factor receptor (Culouscou *et al.*, 1993; Parnell *et al.*, 1995; Xia and Serrero, 1998). Localization of the acrogranin receptor in the blastocyst, which could afford insights regarding acrogranin action on trophectoderm and ICM cells, awaits its molecular identification and characterization.

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