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Conditioned Media of ASC-17D Sertoli Cells Induce G1-Growth Arrest of DU145 Human Prostate Cancer Cells

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We studied the effects of ASC-17D rat Sertoli cell-conditioned media (rSCCM) on the proliferation of the DU145 prostate cancer cells. rSCCM was prepared from ASC-17D cells cultured in DMEM/F-12 serum-free media at a nonpermissive temperature of 40°C, which is the condition for the high expression of clusterin. We found that rSCCM could inhibit the proliferation of DU145 cells by arresting the cell cycle in the G1 phase in a dose-dependent manner. This growth arresting activity was abolished by boiling rSCCM for 5 min. The G1 growth-inhibiting activity of rSCCM was also detected in other prostate-originated cancer cells examined (i.e., LNCaP and PC-3) but not in other cells (ASC-17D, HepG2, SK-N-SH, and NIH3T3). Western blot analysis of partially purified growth inhibiting fractions with the clusterin antibody showed that the cytostatic factor in rSCCM was not clusterin. This cytostatic factor was semipurified by DEAE-Sepharose, ammonium sulfate precipitation, and Phenyl-Sepharose column chromatography, and was estimated to have a molecular weight of 88 kDa by Sephacryl S-300 gel filtration.

Keywords: Conditioned media, DU145 prostate cancer cell, Growth arrest, Sertoli cell.

Introduction

Clusterin was first identified from the ram rete testes fluid as an aggregating factor (Blaschuk *et al.*, 1983; Fritz *et al.*, 1983). Since then it has been purified in various tissues and was suggested to have roles in a number of biological processes based on its tissue expression pattern and/or biochemical properties (Jenne and Tschopp, 1992). Upregulated expressions of clusterin have been reported in apoptotic cell death of certain tissues in response to hormonal modulation or injury, such as, prostate involution by castration, mammary gland involution, neurodege-nerative diseases, and kidney injury (Jenne and Tschopp, 1992; May and Finch, 1992; Tenniswood *et al.*, 1992). Purkinje cells have been shown to express high levels of clusterin transcripts prior to apoptotic cell death induced by the *lurcher* gene induction (Norman *et al.*, 1995). Recently, Michel *et al.* (1997) presented evidence that the clusterin gene expression is correlated with neuronal apoptosis in the lesioned olfactory mucosa. Such a tight correlation between clusterin upregulation and apoptotic cell death suggested the possibility that clusterin could be directly involved in the process of apoptotic cell death. In contrast, there were also many reports showing the constitutive expression of clusterin in diverse tissues including Sertoli cells and many epithelial boundary cells of esophagus, epididymus, gall bladder, and biliary ducts (Griswold *et al.*, 1986; Aronow *et al.*, 1993). In addition, other investigators reported that clusterin was not expressed in the developmental apoptotic cell death of the central nervous system (Garden *et al.*, 1991) nor in the apoptotic thymocytes (French *et al.*, 1992). Furthermore, Sensibar *et al.* (1995) and Humphreys *et al.* (1997) showed that TNF-alpha cytotoxicity could be protected by an overexpression of clusterin in LNCaP prostate cancer cells and L929 murine fibrosarcoma cells, respectively. These reports suggested that clusterin induction could be a stress-associated response for the protection of, rather than the inducer of, cell death. Thus, we questioned whether the cytoprotective or cytotoxic function of clusterin could be related with the concentration of clusterin. In an attempt to study the function of clusterin, we tried to purify

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clusterin from the secreted conditioned media of rat ASC-17D Sertoli cell culture. The rat ASC-17D Sertoli cell-line was immortalized from the sexually mature Sprague-Dawley Sertoli cell primary culture using the SV40 temperature-sensitive mutant virus of Roberts *et al.* (1995). The ASC-17D Sertoli cells grew rapidly at the permissive temperature (32°C) and did not express clusterin. However, at the nonpermissive temperature (40°C), these cells ceased dividing and began to express Sertoli cell-specific proteins, including clusterin. For the purification of clusterin, we collected the conditioned media secreted from the ASC-17D Sertoli cells at conditions that allowed the high expression of clusterin, and concentrated by ultrafiltration using the Amicon YM10 membrane. During the course of our studies on Sertoli cell secreted clusterin, we noted that the clusterin-enriched rSCCM could inhibit the proliferation of human DU145 prostate cancer cells. In this study, we present the partial purification of the growth inhibiting factor in rSCCM that can inhibit the proliferation of human DU145 prostate cancer cells.

Materials and Methods

Cell culture The rat ASC-17D Sertoli cells were kindly provided by Dr. Kenneth Roberts, University of Minnesota, Minneapolis, USA and maintained in a culture described previously (Roberts *et al.*, 1995). The ASC-17D cells were seeded and allowed to grow up to 80–90% confluency in DMEM/F-12 medium supplemented with 4% fetal bovine serum (FBS) and 1% antibiotic-antimycotic at the permissive temperature of 32°C. The cells were then washed twice with phosphate buffered saline (PBS) and incubated further at the nonpermissive temperature of 40°C for 2 d in the serum-free DMEM/F-12 medium. The serum-free culture medium was centrifuged at 1000 × *g* for 10 min to remove cell debris and stored at −20°C. The human prostate cancer cell lines (DU145, LNCaP, PC-3) were kindly obtained from Dr. Han Soo Lee (KangWon National University, Korea), and the HepG2 hepatoblastoma, SK-N-SH neurolastoma cell lines were purchased from the Korean Cell Line Bank (Seoul National University, Korea).

Northern and Western blot analyses The total amount of RNA was isolated from the ASC 17D Sertoli cells at various time points after raising the temperature from 32°C to 40°C by the acid guanidinium thiocyanate and phenol/chloroform extraction methods as described by Chomczynski and Sacchi (1987). The total RNA samples (10 μg) were fractionated in a 1.2% agarose gel containing 0.67 M formaldehyde, and transferred to a Hybond-N+ nylon membrane (Amersham, IL). The membrane was baked at 120°C for 30 min and hybridized with [α-^{32}P]UTP labeled clusterin cRNA riboprobe in the hybridization buffer previously described (Min *et al.*, 1998). After an overnight hybridization, the membrane was washed twice in 0.5× SSC and 0.1% SDS for 15 min each, and once more stringently in 0.1× SSC and 0.1% SDS at 65°C for 30–45min. The membrane was then exposed to an X-ray film with an intensifying screen at −80°C for 2 d. For the Western blot analysis, the serum-free culture medium was collected at various time points after switching to the nonpermissive temperature of 40°C and concentrated 20-fold using Centricon-P10 (Amicon, USA). Protein samples (20 μl) were separated by nonreducing 10% SDS-polyacrylamide gel electrophoresis and then transferred onto the PVDF membrane. The membrane was then incubated with an anti-clusterin antibody and proteins were visualized using the ECL detection kit (Amersham, Illinois, USA).

Cell proliferation assay Approximately 3×10^3 DU145 cells in 0.1 ml of DMEM containing 5% FBS were plated to each well of the 96-well culture plates. After attachment, the cells were further incubated with 10 μl of rat ASC-17D Sertoli cell-conditioned medium (rSCCM) for 48 h. The cells were then treated with 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) stock solution (5 mg/ml in PBS) for 4 h. The formazan crystal produced by cellular dehydrogenases was resolved in 0.1ml of cell lysis buffer (10% SDS in 0.01N HCl) overnight, and absorbance at 600 nm was read using the ELISA plate reader.

Flow cytometric analysis of cell cycle kinetics 3×10^6 cells were centrifuged at 1000 rpm for 10 min at 4°C. The centrifuged pellets were suspended in 10 ml sample buffer (1g/l glucose in PBS, filtered through a 0.22 μm filter) and washed twice. The cells were then resuspended in the same volume of sample buffer. The cell number was adjusted to 1–3 × 10^6 using the sample buffer and centrifuged at 1000 rpm for another 10 min at 4°C. The centrifuged pellet was suspended by 70% ethanol and fixed overnight at 4°C. The fixed cells were briefly vortexed and centrifuged at 3000 rpm for 5 min. The ethanol was discarded, and the pellets were stained with 1 ml of propidium iodide (PI) solution (50 μg/ml PI in sample buffer containing 100 U/ml of RNase A). Before analysis by flow cytometry, each sample was incubated for 30 min at room temperature. The PI–DNA complex in each cell nucleus was measured with FACS (Beckton & Dickinson Co., USA). The rate of the cell cycle within G1, S, and G2/M phases was determined by analysis with the computer program CellFIT.

Partial purification of cytostatic factor All purification steps were carried out at 4°C. The buffer used for the purification of a cytostatic factor was 20 mM potassium phosphate (pH 7.5). The serum-free conditioned media of Sertoli cell (ASC-17D) was applied to a DEAE-Sepharose column. After washing the column with the equilibration buffer, elution was conducted with a linear salt gradient from 0 to 0.5 M NaCl in the equilibration buffer. The active fractions were collected and adjusted to 30% saturation with solid $(NH_4)_2SO_4$ and stirred at 4°C for 3 h. The solution was then centrifuged and the pellet discarded. The supernatant was brought to 85% saturation with solid $(NH_4)_2SO_4$ and treated as described above, except that the pellet was retained and dissolved in an equilibration buffer containing 1 M $(NH_4)_2SO_4$. The dissolved solution was then applied to the Phenyl-Sepharose column, and equilibrated with buffer containing 1 M $(NH_4)_2SO_4$. After washing the column with the same buffer, elution was performed with a reverse linear salt gradient from 1 to 0 M $(NH_4)_2SO_4$ in the equilibration buffer. The active fractions were collected, concentrated by an ultrafiltration (Amicon's stirred

cell, YM-10) and dialyzed against the equilibration buffer. The concentrated sample was used for all other studies.

Molecular weight determination Gel filtration on Sephacryl S-300 column was used to estimate the molecular weight of a partially purified cytostatic factor as described previously (Kim *et al.*, 1995). The column (1 × 100 cm) was equilibrated with PBS. A sample after partial purification on DEAE-Sepharose, ammonium sulfate fractionation, and Phenyl–Sepharose was applied. Fractions of 0.9 ml each were collected and assayed for cytostatic activity as described before. The standard markers were as follows: Thyroglobulin (669,000), γ-globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B_{12} (1350).

Results and Discussion

Clusterin expression of ASC-17D Sertoli cells Immortalized rat ASC-17D Sertoli cells have been known to differentiate and express Sertoli cell-specific proteins including clusterin when the culture temperature was switched from the permissive temperature of 32°C to the nonpermissive temperature of 40°C (Roberts *et al.*, 1995). Using the ASC-17D Sertoli cell line, we have found that the clusterin transcript could be induced at high rates, even in the serum-free DMEM/F-12 media at 24 h after raising the temperature to 40°C (Fig. 1A). This level of clusterin expression in the serum-free media was similar to that in the DMEM/F-12 media supplemented with 4% FBS. We have also noted that the clusterin polypeptide was secreted from the ASC-17D Sertoli cells and accumulated in the serum-free media after raising the temperature to 40°C (Fig. 1B). The secretory function of the ASC-17D Sertoli cells, even in the serum-free medium, could lead us to easily purify clusterin or other growth regulatory factors. Clusterin has been known to be induced during programmed cell death/apoptosis. However, little is known on the role of clusterin in the process of cell death. In this study, we have harvested the serum-free conditioned media secreted from ASC-17D Sertoli cells under conditions that allow the high levels of expression of clusterin, and concentrated the secreted media. Ultrafiltration using the Amicon YM10 membrane was done to purify clusterin in order to study and understand its function.

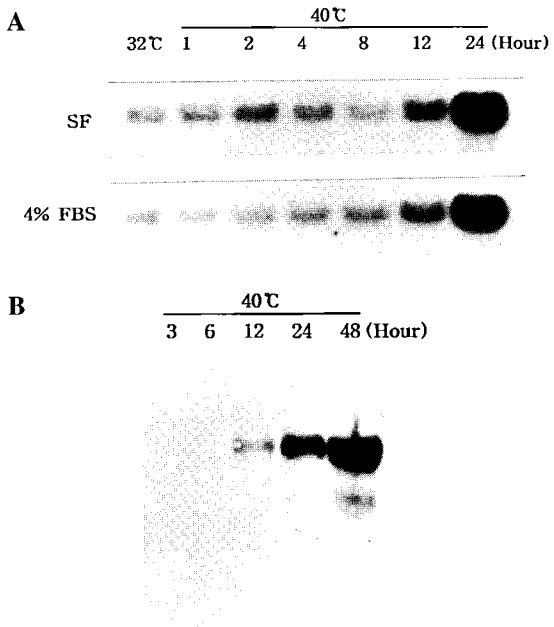

Fig. 1. Northern blot (A) and Western blot (B) analyses of clusterin in ASC-17D rat Sertoli cells. ASC-17D cells were cultured at a permissive temperature (32°C) and switched to a nonpermissive temperature (40°C) for various time periods with or without fetal bovine serum (4%).

Growth inhibiting activity of clusterin-enriched rSCCM Clusterin gene expression is highly induced in degenerating prostate epithelial cells of the experimentally castrated rats (Leger *et al.*, 1987). To understand the role of clusterin in the involutionary process of prostate epithelial cells, we have examined the growth inhibitory activity of clusterin-enriched rSCCM on the human DU145 prostate cancer cells, using direct cell counts and MTT assay. The serum-free conditioned media collected from ASC-17D Sertoli cells could inhibit the proliferation of human DU145 prostate cancer cells in a dose-dependent manner (Fig. 2). Boiling the rSCCM for 5 min could abolish the growth inhibitory activity on DU145 cells, suggesting that anti-proliferative factor(s) in rSCCM was heat-labile. The lactate dehydrogenase (LDH) released from DU145 cells was measured to examine whether cell death could be induced by rSCCM treatment. The LDH activity was lower in the culture medium of DU145 cells treated with rSCCM (50 μg/ml). There was also no significant increase in the number of DU145 cells treated with rSCCM (50 μg/ml) for 3 d (Fig. 3). In addition, we could not detect any DNA fragmentation, a characteristic biochemical feature in apoptosis, in the DU145 cells treated with concentrated rSCCM for 3 d (data not shown). These results suggest that the growth inhibiting effect of rSCCM on DU145 prostate cells are mediated primarily by cytostatic mechanisms rather than cell death. The *in vitro* growth inhibiting activity of rSCCM was examined in DU145, LNCaP, PC-3 human prostate cancer cells, HepG2 hepatoblastoma, NIH3T3, and SK-N-SH neuroblastoma cells. Interestingly, rSCCM preferentially inhibited the proliferation of prostate-originated DU145, LNCaP, and PC-3 cancer cells (Fig. 4). We can speculate that the specific receptor for the cytostatic factor may exist in prostate cells only, but the specificity of the rSCCM-induced growth arresting activity for prostate-originated cells has not been examined yet. Further investigations will be necessary to determine whether a purified factor from rSCCM can still have cytostatic activity for the prostate-originated cells only.

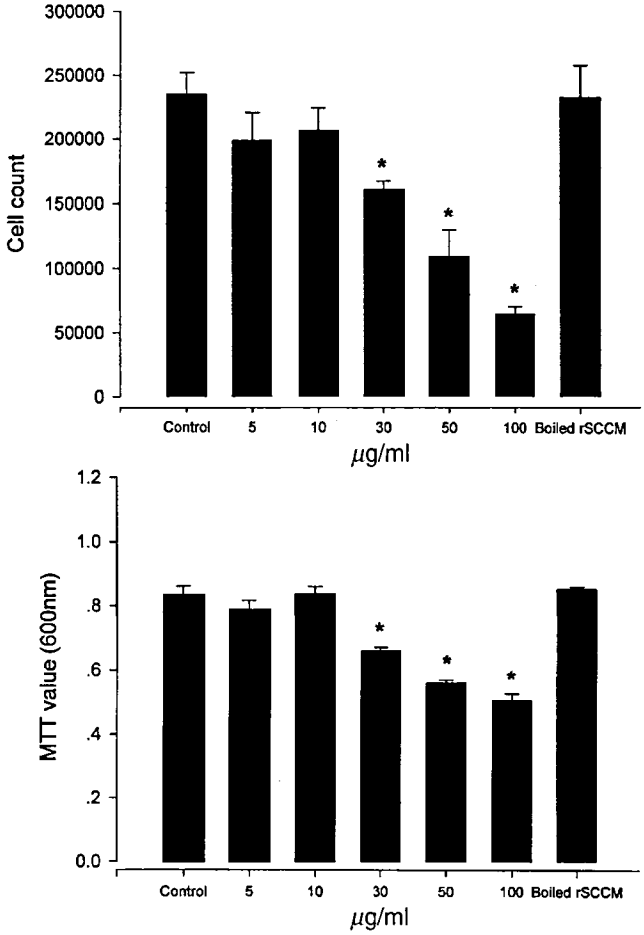

Fig. 2. The effect of rat Sertoli cell-conditioned media (rSCCM) on the DU145 human prostate cancer cell proliferation. The rSCCM from ASC-17D rat Sertoli cells cultured in DMEM/F-12 serum-free media at a nonpermissive temperature (40°C) induced the growth inhibition of DU145 cells in a dose-dependent manner. Boiling the rSCCM for 5 min abolished the growth inhibiting activity on the DU145 cells. The data are mean ± SE from three different experiments. * indicates $p < 0.05$, in comparison to the control values.

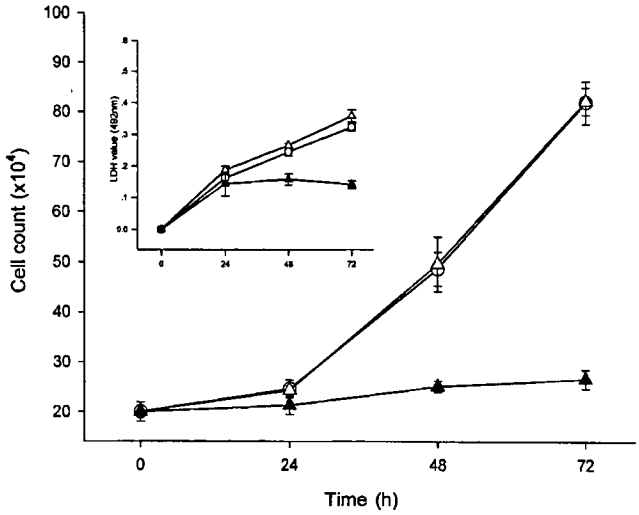

Fig. 3. The effect of rat Sertoli cell-conditioned media (rSCCM) on DU145 human prostate cancer cell proliferation for the various incubation periods. Cell count and LDH (inset) values of the DU145 cultured in the presence of rSCCM (50 μg/ml; —▲—), boiled rSCCM (50 μg/ml; —△—), and PBS (—○—) were measured for 3 d. The values are plotted as mean ± SE from three different experiments.

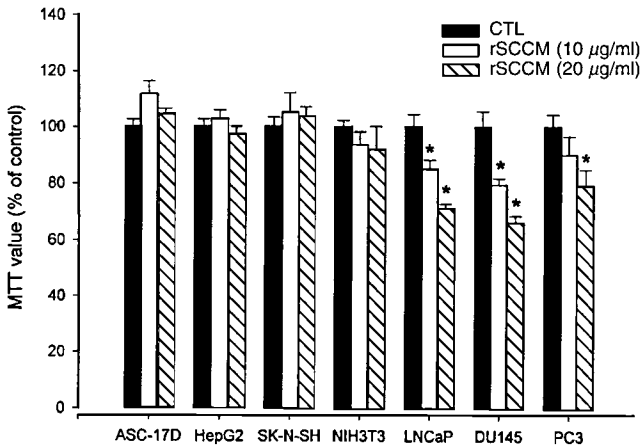

Fig. 4. The effect of rat Sertoli cell conditioned media (rSCCM) on several different cell proliferations. Prostate-originated cancer cells (DU145, PC-3, and LNCaP) are more responsive to growth inhibition by rSCCM than other tested cells (ASC-17D, HepG2, SK-N-SH, and NIH 3T3). The data are mean ± SE from three different experiments. * indicates $p < 0.05$, in comparison to the control values.

Cytostatic factor in rSCCM was not clusterin

Clusterin has been known to have cytoprotective roles in TNF-alpha induced apoptotic cell death (Sensibar *et al.*, 1995; Humphreys *et al.*,1997). These reports, and the growth arresting activity of clusterin-enriched rSCCM, led us to speculate that the cytoprotective function may be a result of the cytostatic activity of clusterin. We performed Western blot analysis using the clusterin antibody in the crude rSCCM and a semipurified growth inhibitory fraction in order to examine whether this growth inhibitory molecule is clusterin or not. The same amount as that used in a growth proliferation assay of DU145 prostate cells was loaded on SDS-PAGE and analyzed for the Western immunoblot with a clusterin antibody. After the semi-purification from crude rSCCM by ammonium sulfate precipitation, DEAE-Sepharose, and Phenyl-Sepharose column chromatography, we could not detect clusterin immunoreactivity in the growth inhibiting fraction (Fig. 5). However, the more potent growth inhibiting activity on DU145 prostate cells was observed in the semipurified fraction rather than the crude rSCCM. This result suggests that other factor(s) rather than clusterin in rSCCM has a growth inhibitory activity on the DU145 prostate cells.

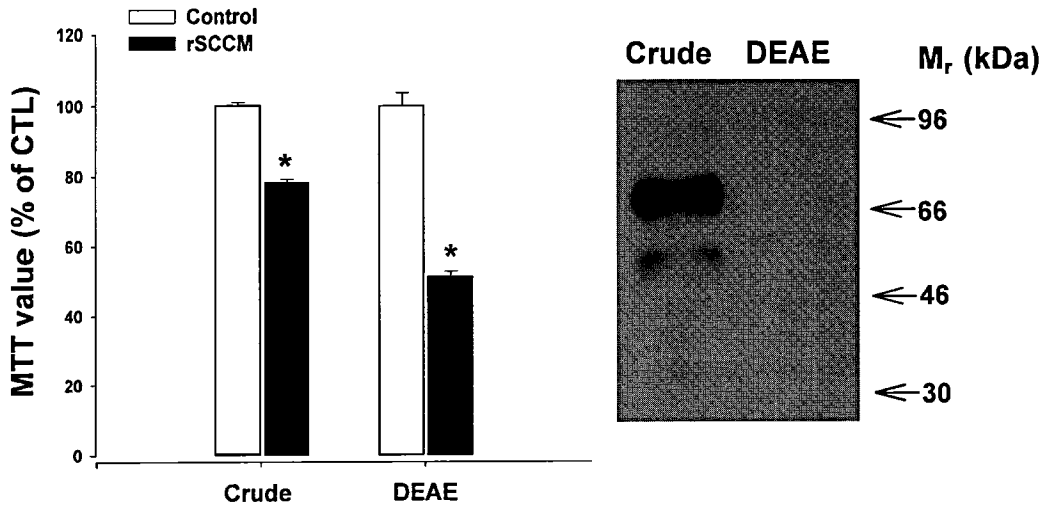

Fig. 5. The anti-proliferating activity and Western blot analysis of clusterin in both crude and semipurified conditioned media. A comparison of the crude and semipurified samples from the rat Sertoli cell conditioned media (rSCCM) by ammonium sulfate precipitation and DEAE-Sepharose column show that clusterin (sulfated glycoprotein-2) does not have growth inhibiting effects on DU145 prostate cells.

Flow cytometry analysis of cell cycle Growth inhibition of DU145 cells by rSCCM appears to be mediated primarily by cytostatic effects rather than by cell death. We examined the cell cycle distribution for the growth arrest of DU145 prostate cells induced by factor(s) in rSCCM. The number of cells in the G1 phase was significantly increased with the simultaneous reduction of cells in the S phase in DU145 prostate cells cultured with rSCCM for 4 d (Fig. 6). This result suggested that the transition from the G1 to S phase was blocked in rSCCM-treated DU145 prostate cells. There was no significant increase of the sub-G1 fraction in the DU145 prostate cells treated with rSCCM, suggesting that the apoptotic cell death is not induced by rSCCM. Further studies on the expression of cell cycle regulatory proteins using purified cytostatic factor(s) are necessary to clarify the cell cycle restriction point.

Semipurification and molecular weight of cytostatic factor The growth inhibiting activities of column fractions were routinely quantified by measurements of formazan produced by cellular dehydrogenases (MTT assay) as described in Materials and Methods. However, a visual inspection of the cultured cells also provided a rapid and sensitive assessment of activity during the purification process. To characterize the growth inhibitory factors present in the rSCCM, 7 liters of serum-free conditioned media of ASC-17D Sertoli cells was applied to a DEAE-Sepharose column and fractionated with a linear salt gradient from 0 to 500 mM NaCl. The active fractions were eluted at about 300 to 400 mM NaCl and precipitated with 85% ammonium sulfate. The growth inhibitory fraction dialyzed against 1 M $(NH_4)_2SO_4$ was

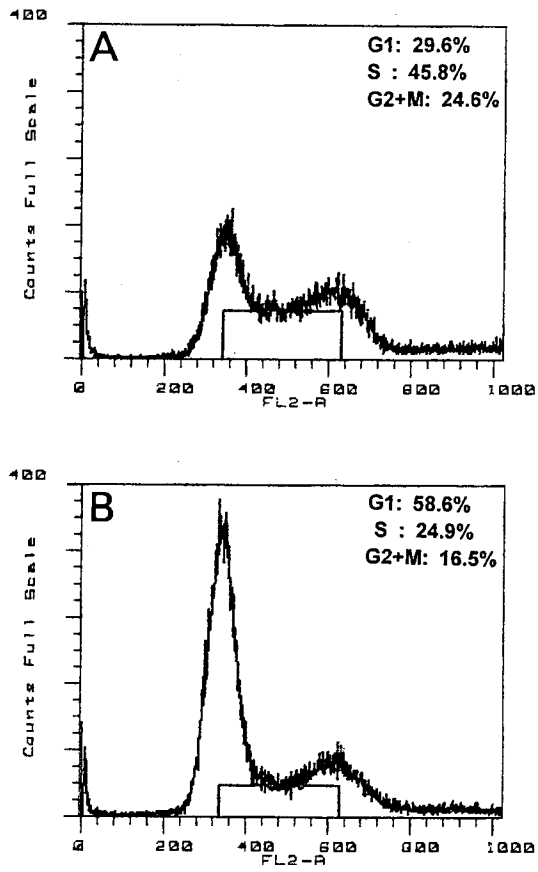

Fig. 6. Rat Sertoli cell-conditioned media (rSCCM) induce a G1 arrest in the cell cycle. The DU145 cells were cultured at a concentration of 3×10^6 in the absence (A) or presence (B) of 50 μg/ml rSCCM. After 4 d, the cells were stained with propidium iodine and the DNA content was assessed by flow cytometric analysis.

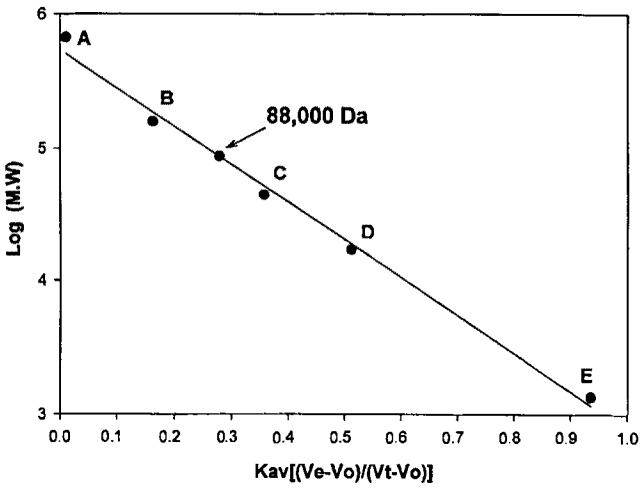

Fig. 7. Determination of the molecular weight of the cytostatic factor by calibrated Sephacryl S-300 gel filtration. A cytostatic factor from rSCCM has been semipurified by ammonium sulfate precipitation, DEAE-Sepharose, and Phenyl-Sepharose. The standard markers were A: Thyroglobulin (669,000), B: g-globulin (158,000), C: ovalbumin (44,000), D: myoglobin (17,000), and E: vitamin B_{12} (1350).

then applied to Phenyl-Sepharose column, and eluted with a reverse salt gradient from 1.0 to 0 M $(NH_4)_2SO_4$. The growth inhibiting activity was separated into a major peak at about 200 mM $(NH_4)_2SO_4$. In order to determine the molecular weight of the major cytostatic factor, a concentrated growth inhibiting fraction was applied to the Sephacryl S-300 gel filtration column. The agent of the growth arresting activity for the DU145 prostate cells was determined to have a molecular weight of 88 kDa, which is larger than that of clusterin (Fig. 7). Sertoli cells are in close contact with germ cells in the mammalian seminiferous tubules and have been known to secrete a variety of proteins that regulate the proliferation of spermatogonia and their differentiation to spermatozoa (Lamb, 1993; Avallet *et al.*, 1994). In this dynamic process of spermatogenesis and other situations such as the depletion of growth factors, hormonal changes, and chemotherapy, germ cells are known to undergo apoptosis (Meistrich, 1993; Bartke, 1995; Hikim *et al.*, 1995). Sertoli cells are also known to secrete factors that induce apoptotic cell death of germ cells as well as having immunosuppressive effects (De Cesais *et al.*, 1992; Lee *et al.*, 1997). The cytostatic factor in rSCCM has a different molecular weight with known growth inhibiting or apoptosis inducing factors such as the Fas ligand, TGF-beta, clusterin, and other cytokines. Further purification and identification of the cytostatic factor in rSCCM will be helpful in understanding the function of Sertoli cells.

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