Culture Shock

SELECTIVE UPTAKE AND RAPID RELEASE OF A NOVEL SERUM PROTEIN BY ENDOTHELIAL CELLS IN VITRO

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A novel protein has been purified from fetal calf serum and from serum-free bovine aortic endothelial cell conditioned culture medium. This protein consists of a single polypeptide chain of reduced M, 70,000 (70K protein) and was isolated from bovine serum albumin and other proteins by ion-exchange chromatography and immunoabsorption on Sepharose-coupled anti-70K protein antiserum. The 70K protein was shown to be structurally and immunologically distinct from bovine serum albumin and from the first protein, nidogen by one- and two-dimensional peptide mapping, amino acid analysis, and enzyme-linked immunosorbent assay and/or immunoblotting.

The 70K protein was located in endothelial cell cytoplasmic granules of irregular size and distribution. Metabolic radiolabeling studies showed that the 70K protein was a metabolic product of these cells, its cytoplasmic location was due to a selective uptake from the fetal calf serum in which the cells were initially grown. After subconfluent cultures of endothelial cells were shifted to serum-free medium, nearly 80% of the total 70K protein that was measurable in the medium was released between 0 and 20 min. Moreover, sparse, rapidly proliferating cells released approximately 18-fold more 70K protein within 2 min as compared to dense, nonproliferating cultures. The concentration of 70K protein in fetal calf serum was estimated to be 400–600 µg/ml. Proliferating bovine aortic endothelial cells, 24 h after plating at an intermediate density, released approximately 250 pg of 70K protein/cell within the first 20 min after exposure to serum-free conditions. The data provide evidence for a novel protein in serum which is selectively internalized by endothelial cells in vitro and which in turn is released rapidly under conditions such as osmotic imbalance due to serum removal, or during periods of cellular proliferation, conditions which we term "culture shock."

The establishment of eucaryotic cells in tissue culture has permitted direct examination of selected, differentiated properties as manifested by homogeneous cellular populations. In particular, studies on vascular endothelial cells have been facilitated by the use of somewhat specialized conditions designed to maintain the several morphologically and biosynthetically unique phenotypes during in vitro propagation (1, 2). Recent studies have confirmed, however, that certain metabolic properties characteristic of the endothelium become significantly altered as a result of subcultivation (3).

Fetal calf serum contains a number of factors that have been directly implicated in cellular phenotypic modulation. The use of serum-free culture conditions as described by Barnes and Satô (4) demonstrates for some cell types that the presence of certain serum components causes significant qualitative and quantitative changes in protein biosynthesis (5). Cellular proliferation, a requisite property for subcultivation, is ensured by the presence of mitogens and growth factors in serum. Since in vivo the uninjured endothelium generally exhibits an extremely low replication rate (6), excessive proliferation in vitro, in the presence of platelet mitogens, could mimic a form of cellular injury (see Refs. 7 and 8 for discussion on this point). Endothelial cells, both in vivo and in vitro, undergo fluid endocytosis. Quantitative measurement of the endocytotic rate in bovine aortic endothelial (BAE) cells in vitro showed significant increases in growing as compared to quiescent cultures and in experimentally wounded cells that were migrating and proliferating in response to this injury (9). The effects of other types of endothelial injury, e.g. cellular exposure to free fatty acids (10), calcium ionophore (11, 12), and oxygen radicals (12) have been measured by the net transfer of albumin across endothelial cell monolayers. An increase in cellular permeability of this serum protein was associated with the injurious agents and was in turn accompanied by osmotic imbalance in cell shape and actin filaments. The rate of albumin transfer was subsequently reduced when the cells were re-exposed to tissue culture medium containing fetal calf serum (FCS).

Cells which have been isolated from a tissue matrix and propagated in vitro are the victims of "culture shock." Various factors can be considered as contributors to cellular injury in vitro: sparse plating density, cellular migration and proliferation, exposure to serum or other wound-associated factors, incubation with radioliposome, imposition of a plastic or other alien substratum, and removal of nutritional/survival components required by the cells. During a study of stress-related protein secretion (57), we observed a protein of M, 70,000 (70K protein) in BAE cell culture medium. It was derived

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The abbreviations used are: BAE, bovine aortic endothelial; 70K and 43K proteins, proteins with M, 70,000 and 43,000, respectively; αFP, α-fetoprotein; BSA, bovine serum albumin; FCS, fetal calf serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DEEM, Dubuque's modified Eagle's medium; DTT, dithiothreitol; Tris-saline, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5; PBS, phosphate-buffered saline buffer, pH 7.2; ELISA, enzyme-linked immunosorbent assay.
from the FCS in which the cells were initially cultured and represented a unique and novel protein that was structurally different from bovine serum albumin (BSA), α-fetoprotein (AFP), and vitronectin. This 70K protein was released rapidly from BAE cells under conditions of serum deprivation or proliferation. We propose that the selective uptake and rapid release of this novel 70K protein from BAE cells in vitro is indicative of culture shock and concomitant cellular injury.

MATERIALS AND METHODS

Cell Culture—Endothelial cells (provided by Dr. S. Schwartz, University of Washington, Seattle, WA) were obtained from adult bovine aorta by scraping the intimal layer gently with a scalpel, as described by Gajdusek and Schwartz (15). BAE cells were also alternatively prepared by collagenase treatment of isolated vessels (14) and were provided by Dr. J. Harian (University of Washington, Seattle, WA). The cells were grown in either Waymouth's or Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) containing antibiotics and 1%, by volume, fetal calf serum (lot 100424, Hyclone Laboratories, Sterile Systems, Inc., Logan, UT) and were subcultured at a 1:3 split ratio in equal volumes of trypsin (0.2% solution, Gibco Laboratories) and EDTA (0.02% solution, pH 7.5, MCB, Cincinnati, OH). Several different strains of cells, ranging in passage number from 6-12, were used during the course of the experiments. All cultures reached confluence within 3 days after plating and, by morphologic criteria, were homogeneous and not senescent.

Chromatographic Procedures—70K protein was purified from both FCS (lot 100424, Hyclone) and serum-free BAE cell culture medium. Chromatography on Sephadex G-200 (Pharmacia), DEAE-cellulose (DBS2, Whatman), and heparin-Sepharose CL-6B (Pharmacia) was performed as previously described (15). DEAE-Affi-Gel® Blue (Bio-Rad) (8 ml) was equilibrated at 4 °C in 0.02 M K2HPO4 buffer, pH 8.0. In a typical experiment, 1 ml of FCS was dialyzed against the equilibration buffer and applied to the column; following a 40-ml wash to remove unbound material, bound protein was eluted with equilibration buffer containing 1.4 M NaCl, at a flow rate of 40 ml/min. Column effluents were monitored by absorbance at 230 nm.

Antiserum 1256, which was raised initially against a BAE cell glycoprotein of M, 43,000 (45K protein) (15), but which also contained antibodies reactive with the 70K protein, was dialyzed against 0.1 M NaCl, pH 8.3 buffer at 4 °C. 2 ml of antiserum were subsequently depleted of protein in PBS ranging from 50-500 μg/ml. Column effluents were monitored by absorbance at 230 nm.

Results

In earlier studies we had described the biosynthesis of a secreted glycoprotein, of M, 43,000, by BAE and several other mesenchymal cells in vitro (15). This protein exhibited an extremely high affinity for BSA which was dissociable only in the presence of SDS. Nonaffinity-purified antiserum, generated originally to a 43K/BSA/70K complex, was coupled to Sepharose in an attempt to dissociate the 43K protein from its ligand(s). When dialyzed BAE cell-conditioned medium was passed over this column, the major species that bound was neither 43K protein nor BSA, but a protein of apparent

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reduced $M_r$ 70,000 that exhibited a slightly lower mobility than BSA ($M_r$ 67,000-68,000) on SDS-PAGE. This result is shown in Fig. 1A (lane 1). In the absence of reduction, the bound protein displayed a molecular weight of approximately 55,000 (lane 2, -DTT) and therefore appeared to be a single polypeptide chain containing intramolecular disulfide bonds.

At this point we investigated the origin of the 70K protein. Biosynthetic studies carried out under standard conditions in the presence of $[^3H]$proline did not reveal a radioactive band in the molecular weight range of 70,000 after SDS-PAGE analysis of both culture medium and cell layer components (data not shown). To examine the possibility that the 70K protein was a plasma membrane or cell surface component of low abundance or metabolic turnover, we incubated sparse, freshly plated cells with $[^3H]$leucine for 76 h. During this interval, the cells underwent at least two population doublings. Again, a radioactive band of 55,000 proteins (250-500 ng), a rough estimate of the maximum amount of 70K protein in FCS, was not visible by staining with Coomassie Blue. Based on the level of detection afforded by this dye for $M_r$ 50,000 proteins (250-500 ng), a rough estimate of the maximum amount of 70K protein in FCS was 1-2% of the BSA content, or approximately 250-500 mg/ml.

70K protein was also purified directly from FCS by affinity chromatography on Affi-Gel Blue. When the unbound fraction (uFP + 70K protein) and bound fraction (BSA) were analyzed on a 5% separating SDS-polyacrylamide gel, the difference in molecular weight between 70K protein and BSA became more apparent. As shown in Fig. 2 (panel 1), the protein not retained by the resin displayed a lower mobility on SDS-PAGE than either the major protein component in FCS (BSA, bottom panel) or the BSA which was adsorbed by the column and subsequently eluted with 1.4 M NaCl (panel 2). Precise quantitation of the amount of 70K protein in FCS, relative to BSA, was not possible for two reasons: the application of NaCl caused some of the blue dye to leach from the

FIG. 1. Isolation of 70K protein from BAE cell culture medium by antibody affinity chromatography. 2 ml of serum-free conditioned medium from BAE cells (A), or a protein fraction thereof isolated by DEAE-cellulose chromatography (B), was applied to a column of Sepharose CL-4B to which had been coupled antiserum 1256 (see "Materials and Methods"). After several column washes with PBS, bound protein was eluted with a 0.2 M glycine buffer, pH 2.2. Fractions were pooled from several runs and were analyzed by SDS-PAGE on 5/10% composite gels, both before and after reduction with 50 mM DTT. Proteins were visualized by staining with Coomassie Blue. Lanes 1 contain unbound material, and lanes 2 contain protein that was eluted with the 0.2 M glycine buffer. BSA and proteins of $M_r$ 43,000 (43K) and 70,000 (70K) have been identified. In panel A, protein molecular weight standards are shown on the far left, and a sample of fetal calf serum (FCS) is shown in panel B.
column, thereby interfering with the absorbance of the protein at either 230 or 280 nm. In addition, the recovery of BSA from Affi-Gel Blue was 25 ± 5%. Removal of the bound BSA by boiling an aliquot of the resin in Laemmli buffer, followed by SDS-PAGE, showed that this fraction did not differ in molecular weight from that eluted by 1.4 M NaCl (not shown). From the data in Fig. 2, 1.6% of the total BSA content, or 420 μg of 70K protein, was present in 1 ml of FCS (based on 26 mg/ml BSA in FCS and a 100% recovery of 70K protein). This value (420 μg) was within the range estimated for the recovery of 70K protein from BAE cell postculture medium (250–500 μg/ml).

The structure of the 70K protein was shown to be different from that of BSA, αFP, and several other serum proteins. The mobility of purified 70K protein was slightly lower than that of purified BSA (Pentex) or BSA (FCS) in an acid-urea-PAGE system, and a more marked difference was seen in a “native” gel system as described by Ornstein (20) and Davis (21) (data not shown). The most significant difference in mobility between BSA and the 70K protein was observed on Laemmli gels in the absence of SDS and urea. A difference of similar magnitude was seen after molecular sieve chromatography on Sephadex G-200, in which the 70K protein eluted midway between the molecular weight standard proteins BSA (67,000) and ovalbumin (43,000) (data not shown).

Comparative one-dimensional peptide mapping, based on limited cleavage by trypsin and proteinase K, was performed on BSA, αFP, and 70K protein (data not presented). While BSA apparently contains no trypsin-sensitive sites, a single major band (M, 65,000) was produced from αFP, and limited cleavage, with no peptides evident of M, < 10,000, was observed with the 70K protein. In contrast, a discrete set of unique peptides was produced from each protein with proteinase K. Two-dimensional mapping of [125I]peptides produced by complete proteinase K digestion showed a lack of coincidence of several major peptides (Fig. 3I). The overall differences in topology between the maps of BSA (a) and 70K protein (b) did not reveal a convincing homology, even after very long exposures (c and d).

The structural relationship between the 70K protein and several serum or plasma proteins was also studied with immunochromatographic probes. Purified αFP, FCS (containing BSA, 70K protein, and αFP as principal components), and an impure preparation of 70K protein were exposed to anti-αFP antibodies, followed by [125I]-protein A, in a Western blot assay. Fig. 3IB shows the Amido black stain of these samples, and the amounts of αFP in each lane can be seen after the immunostaining reaction (A). αFP was present both in FCS and in the preparation of 70K protein and migrated in this SDS-PAGE system with an apparent M, of 75,000. The major protein component in the 70K preparation (Fig. 3IB, lane 70K) did not react with the αFP antisera (A, lane 70K). In similar immunoblotting experiments, the 70K protein did not react with antisera toward either von Willebrand protein or BSA (data not shown).

In Fig. 4 are summarized data from several ELISAs, all of which were performed with rabbit anti-bovine 70K protein antibodies. This antibody preparation, which had initially been affinity-purified against the 43K protein as bound to BSA and 70K protein, demonstrated no reactivity toward fibronectin, fibrinogen, von Willebrand protein, and thrombospondin (data not presented; 15). In panel A, the antisera was passed over a BSA-Sepharose column and showed maximal reactivity toward 70K protein, a somewhat reduced response to FCS, and a low but residual activity towards BSA.

| Table 1: Amino acid composition of two serum proteins (in residues/1000 residues) |
|---------------------------------|-----------|-----------|
| 70K*                            | BSA*      |
| Asx                             | 95.3      | 100.2     |
| Thr                             | 43.1      | 61.60     |
| Ser                             | 135.7     | 51.58     |
| Glx                             | 130.6     | 131.0     |
| Pro                             | 29.9      | 46.70     |
| Gly                             | 196.3     | 30.97     |
| Ala                             | 64.7      | 77.28     |
| Val                             | 38.0      | 61.60     |
| Ile                             | 37.5      | 23.52     |
| Leu                             | 76.4      | 107.0     |
| Cysb                            | 16.1      | 50.46     |
| Met'                            | 11.7      | 8.01      |
| Tyr                             | 19.4      | 15.51     |
| Phe                             | 33.3      | 49.62     |
| Lys                             | 51.2      | 107.5     |
| His                             | 21.9      | 30.91     |
| Arg                             | 47.3      | 43.74     |

* Serine increased by 10% and threonine increased by 5% to correct for hydrolytic losses. Hydroxyproline and hydroxylysine were absent. Tryptophan was not determined.

* As cysteic acid.

* As methionine sulfone.
When the antiserum was, in addition, absorbed in solution with thrombospondin (which also served as a control for dilution of the antibody with a protein-containing solution), a result similar to that shown in panel A was obtained (panel C). In an effort to eliminate the small fraction of BSA reactive antibodies, the antiserum was absorbed in solution with BSA. As shown in panel B, removal of this reactivity resulted in an approximately 20% decrease in the response of the antiserum to the 70K protein and a slightly decreased reactivity to FCS. We have found by ELISA and immunoblotting that preparations of BSA, as supplied from both Sigma and Pentex, contained small amounts of 70K protein. Similarly, the anti-BSA antiserum fraction, as supplied by Cappel, displayed a low but detectable activity toward the 70K protein. Prior absorption of anti-BSA antiserum with 70K protein abolished this activity, but the initial titer against BSA was retained (data not shown). When the anti-70K antiserum was absorbed in solution with the 70K protein, the antibody population recognized neither 70K protein nor FCS (panel D). Since these ELISAs were not quantitative, we did not determine the amount of the 70K protein in FCS by this procedure. The data provide strong evidence, however, that BSA and the 70K protein are also immunologically distinct.

Further confirmation of the difference between BSA and the 70K protein was provided by amino acid analysis (Table I). There were highly significant differences in at least 4 residues: Ser, Gly, Cys, and Lys. While both BSA and the 70K protein exhibited a high percentage (approximately 20%) of potentially acidic amino acids, BSA contained a higher proportion of residues with bulky, hydrophobic side chains (Val, Leu, Phe, Lys).

Unlike many proteins that have been isolated from serum or plasma, the 70K protein did not bind to heparin-Sepharose (data not shown). It was also distinct from a recently described serum protein of similar molecular weight, vitronectin, by amino acid composition (25). In addition, studies showed that the 70K protein did not promote cell attachment and was unreactive when immunoblotted with anti-bovine vitronectin antiserum.

The distribution of the 70K protein in BAE cells was studied by immunofluorescence and by SDS-PAGE of secreted, cell surface, and cytoplasmic components (Fig. 5). Antibodies specific for the 70K protein (as confirmed by ELISA) were localized to perinuclear granules in BAE cells which had been rendered permeable by prior ethanol treatment (A and B). In the absence of this step, there was no apparent staining of either the cell surface or the substrate-attached material (as seen between cells at subconfluent den-
FIG. 4. ELISA of serum proteins with anti-70K protein antibodies. 70K protein (2.5 μg/ml) (solid line), FCS (10 μg/ml, 1:2500 dilution) (dotted line), and BSA (25 μg/ml) (dashed line), applied to individual microtiter wells, were exposed to anti-70K protein antiserum, and reactivities were monitored by ELISA. A, antiserum, affinity-treated by passage over a BSA-Sepharose column; B, antiserum, absorbed in solution with BSA (the concentration of BSA used to coat the dish was 100 μg/ml); C, antiserum, as A, and also absorbed in solution with thrombospondin (single point at 10⁻⁴ antibody dilution indicates reactivity toward BSA); D, antiserum, absorbed in solution with 70K protein.

FIG. 5. Distribution of 70K protein in BAE cells in vitro. BAE cells were grown on glass coverslips and were fixed and rendered permeable as described under "Materials and Methods." Panel A shows cells exposed to a sample of anti-70K protein antiserum that had been previously absorbed with BSA, thrombospondin, and 43K protein. A 1:100 dilution of this antibody was used for the results shown in panel B. After 30 min, goat anti-rabbit IgG coupled to fluorescein isothiocyanate was added to identify positive immune complexes. In panel C is shown a control exposure with normal rabbit IgG. All exposures were for 2 min. Magnification × 350. Nearly confluent cultures of BAE cells were thoroughly washed and then exposed for 2 h to serum-free DMEM, after which the cells, culture medium, and cell surface were examined for the presence of 70K protein. Proteins were analyzed by SDS-PAGE on a 5/10% composite gel in the presence of DTT and were visualized by staining with Coomassie Blue. D, 5% by volume of clarified culture medium, diluted 1:1 with Laemmli buffer and applied directly to gel; E, after removal of the medium, cells were subjected to a brief trypsin shave, as described under "Materials and Methods," and 12.5% by volume of the total releasate was diluted 1:1 with Laemmli buffer prior to SDS-PAGE; F, after the trypsin shave, cells were solubilized directly in hot Laemmli buffer, and 25% of the total volume was applied to the gel. Mobilities of protein molecular weight standards are shown on the far right. Bands of M, 20,000-30,000 in lane E were present initially in the trypsin solution.
Culture Shock: 70K Glycoprotein

FIG. 6. Release of 70K protein from sparse and dense cultures of BAE cells. One dish each of cells at sparse and confluent densities was washed once with PBS and then exposed to 2 ml of serum-free Waymouth's medium. At 0-, 5-, and 15-min intervals, 35 μl of culture medium was removed from each dish, diluted 1:1 with Laemmli buffer containing 50 mM DTT, and analyzed by SDS-PAGE on a 5/10% composite gel. At 15 min, the cells from each dish were solubilized in 300 μl of Laemmli buffer containing DTT, and 25 μl was subsequently applied to the gel. Proteins were visualized by staining with Coomassie Blue, and quantitative analysis was performed by integration of peak areas following scanning densitometry of the respective gel lanes. I, phase contrast microscopy of sparse (A) and dense (B) BAE cells in vitro immediately prior to addition of serum-free medium. II, densitometry tracings of SDS-PAGE analyses of culture medium protein from sparse (A) and dense cells (B), that was released at 0, 5, and 15 min. The major peak shown corresponds to an Mr of 70,000. Panels C and D are tracings of sparse (C) and dense (D) cell layer proteins after a 15-min exposure to serum-free medium. The entire gel lane has been shown, and protein molecular weight markers of 67,000 (BSA) and 43,000 (ovalbumin) have been included.
Culture Shock: 70K Glycoprotein

FIG. 7. Quantitative analysis of release of 70K protein by BAE cells in vitro as a function of degree of confluence (A) and of time (B). A, the amounts of 70K protein released into the culture medium within 2 min after placing BAE cells in serum-free scanning densitometry of the gel lanes corresponding to 2 min for each cell density. The 70K protein was measured relative to the levels of actin (M, 40,000) in the cell layers, and this value was plotted as a function of cell density (Fig. 7A). There was a greater than 12-fold increase in 70K protein released from sparse cells than from subconfluent cells and an approximately 18-fold increase from sparse over confluent cells. The amounts of 70K protein released from confluent cells were similar between 24- and 2-h postsubculture (fresly plated, FP) dishes (Fig. 7A). When the 70K protein, in arbitrary absorbance units, was plotted directly as a function of cell number, graphs were similar to those in Fig. 7A (data not shown).

Release of the 70K protein as a function of time is illustrated in Fig. 7B. The data points were generated from several different experiments in which cells of intermediate density were utilized. If 0 min represented the time at which the cells were exposed to serum-free medium, greater than % of the 70K protein recovered from the culture medium (up to a maximum of 2 h) was released within the first 20 min. After 5 min, only negligible amounts of the 70K protein could be recovered from the cells (Fig. 7B; see also Fig. 6I).

Collectively, these data indicate that the release of the novel serum protein, of apparent M, 70,000, from BAE cells in vitro was (a) rapid (>80% released within 30 min), and (b) significantly enhanced in sparse, proliferating cultures. In order to measure accurately the levels of 70K protein in the culture medium as a function of time, we found that extremely rapid (<30 s) washes were necessary, prior to addition of serum-free medium, to minimize losses of 70K protein from the cells. Such release occurred regardless of the wash solution used (EDTA, PBS, DMEM, or Waymouth's medium). There appeared to be no reproducible differences in the levels of 70K protein released in either the presence or absence of ascorbate, or with DMEM as compared to Waymouth's medium. The release also appeared to be unaffected by the addition of [H]Pro or [H]Leu, from 1–50 μCi/ml culture medium, to the BAE cells.

Numerical estimates of the amount of 70K protein in FCS, in conjunction with published values for BSA, aFP, and fetuin, have been summarized in Table II. In the absence of a molar extinction coefficient for the 70K protein, we were unable to calculate directly the absolute levels of this protein. However, we could predict within reasonable limits the medium have been plotted for sparse, subconfluent, confluent, and confluent freshly plated cultures. To normalize the levels of 70K protein released to an equivalent number of cells, the 70K protein was expressed relative to the amount of actin (M, 40,000) (70K protein/40K protein) present for each cell density. B, six 30-mm dishes of nearly confluent BAE cells, equivalent with respect to subculture, were washed quickly with PBS and were then incubated with 0.5 ml of serum-free DMEM. After 5 min, 25 μl of medium was removed from one dish and diluted with an equal volume of Laemmli buffer containing 50 mM DTT. The remainder of the medium was then removed from the dish, and the cells were solubilized in 100 μl of Laemmli buffer containing DTT. At the same time, medium containing 70K protein that had accumulated within the first 5 min in the other two dishes was replaced with fresh medium. After 20 min, a 25-μl aliquot of this medium and the cells from which it was removed were analyzed as for the 5- and 20-min time points. The three remaining dishes of BAE cells were trypsinized and counted by hemocytometer. Proteins were analyzed by SDS-PAGE on a 5/10% composite gel and were measured quantitatively by integration of peak areas following scanning densitometry of the gel lanes. The graph shows 70K protein, expressed in arbitrary units, released into the culture medium from 0–30 min. All values represent equivalent numbers of cells.
Table II

| Protein          | Concentration | Fetal age |
|------------------|---------------|-----------|
|                  | mg/ml         | days      |
| I. Albumin       | 11            | 120       |
|                  | 20            | 240       |
|                  | 22            | term      |
| II. α-Fetoprotein| 26 ± 4        | pooled sera|
|                  | <1            | 240       |
|                  | 0.2           | term      |
| III. Fetuin      | 10            | 120       |
|                  | 15            | 240       |
|                  | 10            | term      |
| IV. 70K protein  | 0.6           | pooled sera|
|                  | 0.4           |           |

*Ref. 26, plasma.

Biochemical assay for HyClone fetal bovine serum, lot 100424 (HyClone Laboratories, Logan, UT).

Ref. 27, plasma.

Based on recovery of 70K protein and αFP from Affi-Gel Blue (Fig. 2): a maximum value.

Based on release of 70K protein from BAE cells plated at intermediate density (e.g. Fig. 7B): a minimum value.

amount of the 70K protein based on (a) its recovery from Affi-Gel Blue chromatography, relative to αFP, and (b) its release from BAE cells in culture, relative to BSA. In both cases, the published values for αFP and BSA, as shown in Table II, were used, and a standard curve for the Comassie Blue-staining reaction was generated with BSA over a concentration range of 0.1–100 μg.

Studies by LaI and co-workers (26, 27) have shown in fetal bovine plasma a progressive increase in the concentration of BSA with fetal maturation and a concomitant decrease in the levels of αFP. While the concentration of fetuin did not exhibit progressive changes, at parturition the concentration of BSA was 22 mg/ml, and that of αFP, 0.2 mg/ml (Table II). The FCS used in the present study (Hyclone lot 100424) was pooled from approximately 4000 individuals and contained 26 mg/ml of BSA (Table II). This level is considerably higher than those reported for several other mammalian species, in which the gene for serum albumin is activated immediately prior to birth (28). For this lot of FCS, we calculated a maximum concentration for the 70K protein of 600 μg/ml, based on the recoveries of 70K protein and αFP from Affi-Gel Blue chromatography. A minimum value of 400 μg/ml was derived from the cellular release studies. In addition, 105 cells/30-mm dish released into the culture medium 66 μg of 70K protein (based on standard curve with BSA), within the first 20 min of exposure to serum-free conditions, or 250 pg 70K protein/cell.

These calculations define the limits for the concentration of the 70K protein in FCS, from approximately 400–600 μg/ml. The value, 250 pg of 70K protein released/cell, was reproducible within 10% for cultures of BAE cells at the same density and on the same size dish (containing the same amount of culture medium). When cells grown on 100- or 150-mm dishes (in medium containing 42 or 83 mg of BSA, respectively) were compared to those on 30-mm dishes, values somewhat higher than 400 μg/ml for the concentration of the 70K protein were obtained. This difference was essentially due to the concomitant uptake and release of BSA (up to 20% of the total 70K protein) that was characteristic of cultures containing higher numbers of cells, especially after exposure to serum-free conditions for more than 2 h.

This study provides structural characterization of a novel protein that was purified both from fetal bovine serum and from culture medium conditioned by bovine aortic endothelial cells. The protein is a single, disulfide-bonded polypeptide chain with an apparent molecular weight of 70,000 by SDS-PAGE in the presence of DTT. By peptide maps, amino acid analysis, and immunologic criteria, the 70K protein exhibited a unique primary structure. Its levels in FCS were estimated to be 400–600 μg/ml, and it was not a biosynthetic product of the BAE cells in vitro. An interesting observation was the apparently selective uptake and subsequent release of the 70K protein by BAE cells in culture. Within 20 min after exposure to serum-free medium, the cells released nearly 80% of their total internalized 70K protein, which by immunofluorescence had been irregularly distributed within cytoplasmic granules. Sparse, proliferating cells released, within 2 min, approximately 18-fold more 70K protein per cell than that released by confluent, nondividing cells. The data indicate that a novel protein in FCS is taken up by BAE cells during routine cell culture, and that its rapid release is triggered by some form of culture shock, such as exposure to serum-free conditions or during proliferation from subconfluent densities.

Since BSA (M, 68,000) is the major protein component of FCS, experiments were performed to examine whether there was a structural relationship between BSA and the 70K protein. An effective separation of these two proteins was achieved by chromatography on Affi-Gel Blue. Albumins bind selectively to several dyes, including Cibacron Blue (29), while the 70K protein did not demonstrate this affinity. By several criteria including amino acid composition, one- and two-dimensional peptide mapping, ELISA and Western blotting, and apparent molecular weight by PAGE under both native and denaturing conditions, the 70K protein was structurally distinct from BSA. These experimental data were sufficient to establish that the 70K protein was not an alternate and less prevalent form of serum albumin, such as prealbumin (30), proalbumin (31), glycosylated albumin (32, 33), and variant albumins as have recently been shown in alloalbuminemia and by restriction enzyme fragment length polymorphism (34).

Several other serum proteins, with apparent molecular mass in the range of 70,000 daltons, were found to be different from the 70K protein by amino acid composition, immunologic criteria, affinity for heparin, and/or cell attachment and spreading activity. These proteins include α-fetoprotein (35), vitronectin (25, 36), an M, 70,000 human serum protein as described by Vuento and co-workers (37), heparin cofactor II (38), thrombomodulin (39), hemopexin (40), and leucine-rich α2-glycoprotein (41). Reference tables containing the molecular parameters of purified plasma proteins (e.g. 42) did not provide further possibilities for the identity of the 70K protein. It appears therefore that this protein is both a unique and novel component of fetal calf serum (the lot analyzed in this study was pooled from 4,000 individuals).

At this time the pathway by which albumin (and/or the 70K protein) enters the endothelial cell is not clear. An endocytotic mechanism of transendothelial transport, dependent both on the physiological state of the cell and on the biochemical properties of the macromolecule, is most probable (43, see Ref. 9 for a review). That molecular signals might favor selective internalization of certain serum proteins by the endothelium is suggested by the enhanced transport of glycosylated albumin (32) and uptake of acetylated low-density lipoprotein (44), as compared to the rates observed with the underivatized counterparts of these molecules.
The contribution of endothelial injury to disease processes such as atherosclerosis has provided a rationale for understanding the relationship between cellular behavior and overt cellular injury (7, 45). Hansson and co-workers (46) have shown that both IgG and C3 accumulated in injured endothelial cells from normal and atherosclerotic arteries, and that IgG was bound to vimentin-type intermediate filaments within the cells (47). Studies from several laboratories have also demonstrated selective adsorption of serum proteins to the uninjured endothelial surface in vivo, with a subsequent diminution in cellular permeability to other macromolecules (48). Despite the apparent protective effect of albumin and other circulating proteins on endothelial cells, increased rates of macromolecular (especially albumin) transfer across the endothelium have been correlated with cellular injury. Exposure of endothelial cells to oxidants (12, 49), calcium ionophore (11, 12), and free fatty acids (10) resulted in intracellular accumulation of albumin that was associated with changes in cell shape and cytoskeletal configuration. Injury as a result of cell culture can also have an effect on endocytic rate and macromolecular accumulation. Davies (9) found that the rate of fluid endocytosis in wounded, endothelial monolayers was higher in proliferating and migrating cells. In this regard, the higher levels of the 70K protein associated with sparsely plated, proliferating BAE cells, in contrast to those in confluent and quiescent cultures, might reflect the close relationship between a polarized endocytic cycle and cell locomotion, as proposed by Bretscher (50).

In proliferating cultures of BAE cells plated at intermediate density, approximately 250 pg of 70K protein was released per cell within 20 min after exposure to serum-free medium. This figure is expected to be, in part, a function of in vitro conditions, as serial subcultivation of endothelial cells is accompanied by changes both in cell cycle kinetics and in secretory properties (3). Although we observed a gradual and sustained release of BSA from BAE cells in the absence of serum, the rapid release of the 70K protein (>80% of the total internalized 70K protein within the first 30 min) was quite specific. This result is suggestive of a separate pathway and compartmentalization for the uptake, storage, and release of BSA and the 70K protein. One possibility is that the release of BSA reflects the general osmotic imbalance that occurs when cells are placed in serum-free medium, as has been shown for ovalbumin in capillary endothelium (51). In contrast, release of the 70K protein might be diagnostic for the initial events associated with acute cellular injury: e.g. proliferation, migration, or attachment to plastic mediated by the secretion of extracellular matrix macromolecules. In this regard, acute radiation injury has been shown to potentiate the release of von Willebrand protein, but not of fibronectin, from release of the 70K protein might be diagnostic for the separation of extracellular matrix macromolecules. In this regard, acute radiation injury has been shown to potentiate the release of von Willebrand protein, but not of fibronectin, from cells could be a direct consequence of the actin, myosin, and vinculin reorganization that occurs upon migration of previously confluent endothelial cells into an experimental wound (53).

The presence of the 70K protein in serum raises the possibility that it might be selectively internalized by endothelial cells that have sustained a local injury, such as viral infection (54, 55) or after exposure to certain mitogens (56). Endothelial cells in vitro are the victims of culture shock; some of the metabolic changes that occur in response to serum factors, disruption of osmotic balance, plating at sparse density, or in conjunction with migration, proliferation, and cytoskeletal rearrangement could mimic those observed in vivo. Since the function of the 70K protein is presently not known, the significance of its uptake, apparent storage, and rapid release under situations of cellular stress cannot be fully appreciated. An intriguing possibility is that the 70K protein is post-translationally modified to facilitate rapid transcytosis, and in turn is able to function as a carrier protein for selected biosynthetic products of endothelial cells that are up-regulated in response to stress, such as the 43K protein (57). The response of endothelial cells in particular to environmental vicissitudes is in all probability linked to the regulation of genetic transcription for such stress-induced proteins.

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