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IDENTIFICATION AND PARTIAL PURIFICATION OF A NOVEL TUMOR-DERIVED PROTEIN THAT INDUCES TISSUE FACTOR ON CULTURED HUMAN ENDOTHELIAL CELLS

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SUMMARY: Conditioned medium of a human bladder carcinoma cell line (J82) was found to induce tissue factor synthesis in cultured human umbilical vein endothelial cells (HUVEC). A protein present in the J82 conditioned medium was partially purified by FPLC using a combination of MONO Q and Superose 6 columns. The bladder carcinoma-derived cytokine (BCDC) exhibited a Mr of 22kDa by gel permeation HPLC. Polyclonal antibody against either interleukin-1, tumor necrosis factor, or transforming growth factor-β failed to inhibit the ability of the conditioned medium to induce HUVEC tissue factor activity, suggesting that this tumor cell line secretes a novel cytokine responsible for HUVEC tissue factor induction.

Patients with malignant disease often present clinically with a variety of coagulopathies including thrombosis and disseminated intravascular coagulation(1-3). The physiological mechanism whereby the coagulation system is activated in these patients is unclear, although a recent report by Cozzolino et al (4) provided convincing evidence that isolated leukemic cells from patients with acute nonlymphoblastic leukemia and coagulopathy elaborate interleukin-1 which, in turn, induces procoagulant tissue factor activity in endothelial cells and suppresses anticoagulant activity normally associated with the endothelium (5).

In the present study, we demonstrate that the conditioned medium of the J82 human bladder carcinoma cell line induces procoagulant tissue factor activity on the surface of cultured human umbilical vein endothelial cell monolayers. In addition, the bladder carcinoma-derived cytokine (BCDC) has been partially purified from the J82 conditioned medium. This cytokine appears to be distinct from interleukin-1, tumor necrosis factor and transforming growth factor-β, and may play a role in the perturbation of the hemostatic system **in vivo** observed in some patients presenting with neoplasms.

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MATERIALS AND METHODS

Materials - Protein A-Sepharose CL-4B, MONO Q, Superose 6, and SDS low Mr standard kit were obtained from Pharmacia. Bz-Ile-Glu-Gly-Arg-p-nitroanilide(S-2222) was purchased from Helena Laboratories. Medium 199 (M-199) was obtained from Mediatech, Inc., and fetal bovine serum (FBS) was obtained from Hyclone Laboratories, Inc. Penicillin-streptomycin solution, porcine mucosal heparin, human fibronectin and limulus amebocyte lysate (LAL) were products of Sigma Chemical Co. Endothelial cell growth supplement (ECGS) was obtained from Collaborative Research, Inc. Tissue culture flasks were purchased from Corning. Tissue culture plates (24-well) were obtained from Flow Laboratories. Recombinant human α-interleukin 1 was purchased from Genzyme, Inc. Polyclonal (rabbit) anti-human interleukin-1 IgG and anti-human TNF-α IgG were purchased from Endogen. Polyclonal (rabbit) anti-TGF-β was obtained from R & D Systems,Inc.

Proteins - Human factor X and factor VIIa were purified to homogeneity as described (6).

Gel Electrophoresis - SDS-PAGE was performed in slab gels according to Laemmli (7) using 2.5% polyacrylamide stacking gels and 12.5% polyacrylamide gel resolving gels. Gels were stained with Coomassie Blue G-250 and diffusion destained.

Cell Culture Techniques - Endothelial cells derived from human umbilical cord veins were prepared according to Jaffe et al. (8) as modified by Thornton et al. (9). Endothelial cells were grown to confluence in medium 199 supplemented with 20% FBS, 90 μg/ml of porcine mucosal heparin, 100 μg/ml ECGS,100 μg/ml penicillin-streptomycin in tissue culture flasks (75cm^2) coated with human fibronectin (1 μg/cm^2) in a humidified atmosphere at 37°C and 6%CO_2. Cells were passed using 1% trypsin and grown to confluence in 2 cm^2 24-well plates (Flow Laboratories). Experiments were performed on cells after one passage and grown to confluence in 2 cm^2 wells. Human bladder carcinoma cells (J82) obtained from American Type Culture Collection (ATCC:CCL 222), were grown in T-75 flasks containing minimum essential medium (Eagle) supplemented with 10% heat-inactivated FBS, non-essential amino acids and penicillin-streptomycin. J82 cells were maintained at 37°C in an atmosphere containing 6% CO_2 and 98% relative humidity. J82 cells were removed from the T-75 flasks by trypsinization, and 10^7 cells transferred to a 850 cm^2 plastic roller bottle (Corning) containing 100 ml of medium supplemented with 10% FBS. The roller bottle was filled with air containing 20% O_2 and 5% CO_2, capped, and rotated at 1-2 cycle/min at 37°C. After cultures achieved confluence in medium/FBS, the cells were cultured in serum-free medium. The conditioned medium was harvested every 3 days for a total period of two weeks.

Tissue Factor Assay - The expression of functional tissue factor activity on the endothelial cell surface was measured using a two-stage amidolytic assay for factor Xa generated following incubation of the cells with factor VIIa and factor X. J82 conditioned medium, or partially purified J82 cytokine diluted 10-fold with M-199/20% FBS, was added to the tissue culture wells containing confluent human umbilical vein endothelial cells. After 6 hours of incubation at 37°C, the wells were washed three times with incubation medium (M-199/0.5% BSA/5mM $CaCl_2$) at 25°C. Incubation medium (0.5 ml) was added to each well following the last wash. Human factor VIIa (1 μg/ml final concentration) and human factor X (8 μg/ml final concentration) were then added and incubated at 25°C on a rotating platform(120 cycle/min). After exactly 10 min, 400 μl of the supernatant was removed,added to 10 μl of 500 mM EDTA, and kept on ice. Buffer (50 mM Tris-HCl (pH 8.3)/0.15M NaCl; 500 μl) and S-2222 (1 mM; 100 μl) were added to the test aliquot and the ΔA_{405}/min determined using a recording spectrophotometer (Beckman Model DU-65). Factor Xa concentrations were interpolated from a standard curve of ΔA_{405}/min vs. known factor Xa concentrations.

Molecular weight estimation by gel filtration - Gel filtration HPLC was carried out at room temperature using a Bio-Gel TSK-250 column (BIO-RAD;7.5x300mm) equilibrated with 0.15 M sodium phosphate (pH 6.5) containing 0.1 M NaCl. Samples (250 μl) were applied to the column and eluted at a flow rate of 0.6 ml/min. The column was calibrated with thyroglobulin (Mr 670,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 43,000), soybean trypsin inhibitor (Mr 20,100), and myoglobin (17,000).

Purification of J82 cytokine - Unless stated otherwise, all steps in the purification procedure were performed at room temperature. J82 serum-free conditioned medium (500 ml) was centrifuged (15,000xg; 20 min) to remove particulate debris. The conditioned medium was concentrated ten-fold by ultrafiltration (YM-10 membrane), and dialyzed extensively at 4°C against 25 mM Hepes (pH 7.0). The dialyzed preparation was divided into five-10 ml aliquots, and each aliquot filtered (0.22μ membrane) and applied separately to a MONO Q (HR 5/5) column equilibrated with 25 mM Hepes (pH 7.0) at a flow rate of 0.5 ml/min. Following application and washing, the bladder carcinoma derived cytokine (BCDC) was eluted with 25 mM Hepes (pH 7.0) containing 0.2 M NaCl. Active fractions from each run were combined (total pool volume = 15 ml) and dialyzed at 4°C against 25 mM Hepes (pH 7.0). The dialyzed preparation was re-applied to the MONO Q column equilibrated with 25 mM Hepes (pH 7.0). The column was developed with a 30 ml linear gradient from equilibration buffer to 25 mM Hepes (pH 7.0)/0.3 M NaCl. Fractions containing cytokine activity were pooled and 250 μl-aliquots subjected to FPLC gel permeation chromatography in a Superose 6 (HR 10/30) column equilibrated with 50 mM Tris-HCl (pH 7.5)/0.1 M NaCl at a flow rate of 0.3 ml/min. Fractions containing cytokine activity were pooled and stored at -80°C.

RESULTS AND DISCUSSION

In this paper, we report that a human bladder carcinoma cell line (J82) secretes a cytokine into the conditioned medium that is capable of inducing tissue factor procoagulant activity on the surface of cultured human umbilical vein endothelial cells (HUVEC). Kinetic studies revealed that the J82 conditioned medium achieved maximum ability to induce HUVEC tissue factor after 5 days of growth and remained constant for three additional days. Heat-treatment (100°C; 10 min) of the 7-day J82 conditioned medium completely destroyed its ability to induce HUVEC tissue factor. This activity was non-dialyzed, and stable to ultrafiltration, lyophilization and freeze-thawing. Time course studies indicated that the bladder carcinoma-derived cytokine induced maximal tissue factor activity on the HUVEC surface after 8 hrs of incubation with the endothelial cells and the degree of tissue factor production by the J82 conditioned medium was equivalent to that observed by stimulating the HUVEC with 100 U/ml of interleukin-1. The cytokine produced by the J82 was, however, not immunologically related to IL-1, as pretreatment of the J82 7 day conditioned medium with polyclonal anti-human IL-1 (200 NU/ml) had no effect on the ability of the conditioned medium to induce tissue factor on cultured HUVEC. Similar negative results were obtained with polyclonal anti-human tumor necrosis factor (500 NU/ml) and anti-human transforming growth factor-β (100 μg/ml). Inasmuch as endotoxin has been shown by several laboratories to induce HUVEC tissue factor activity, we examined the J82 conditioned

medium for the possible presence of endotoxin. By the limulus amebocyte lysate assay, no endotoxin was found in the day 7 J82 conditioned medium (limit of detection = 0.05 ng/ml). Thus, the tissue factor inducing activity of the J82 conditioned medium did not appear to be due either to endotoxin or a cytokine immunologically related to IL-1, tumor necrosis factor or TGF-β.

Based on the above results, we attempted to isolate the bladder carcinoma-derived cytokine (BCDC) from 7 day serum-free J82 conditioned medium. Preliminary studies indicated that BCDC failed to bind to a MONO-S FPLC column equilibrated with 25 mM Hepes (pH 7.0), but did bind quantitatively to a MONO-Q column equilibrated with this buffer. Progressive stepwise elution of MONO-Q bound BCDC revealed that it eluted from the MONO-Q column between 0 and 0.2 M NaCl. Accordingly, 10 ml aliquots of 10-fold concentrated J82 serum-free conditioned medium were applied to a MONO-Q column equilibrated with 25 mM Hepes (pH 7.0). Following a brief wash, BCDC was eluted with 25 mM Hepes (pH 7.0) containing 0.2 M NaCl. The majority of the applied protein was then eluted with equilibrating buffer/1 M NaCl. The BCDC 0.2 M NaCl eluate were then dialyzed, reapplied to the MONO-Q column and BCDC eluted with a linear NaCl gradient from 25 mM Hepes (pH 7.0) to 25 mM Hepes (pH 7.0)/0.3 M NaCl. Figure 1 demonstrates a typical gradient elution profile for BCDC on MONO-Q FPLC. Two peaks of tissue factor inducing activity were consistently found;

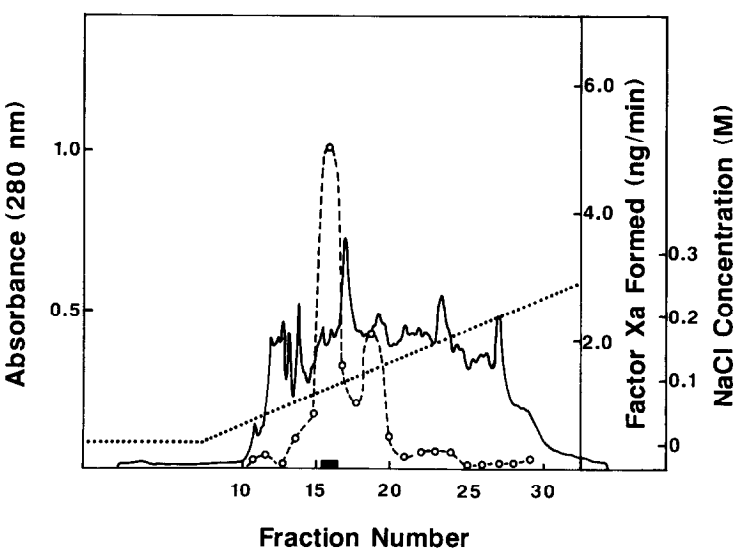


Figure 1: Elution pattern of bladder carcinoma-derived cytokine from MONO-Q FPLC. Protein was eluted from the column (HR 5/5) with a 30 ml linear gradient of NaCl from 25 mM Hepes (pH 7.0) to 25 mM Hepes (pH 7.0)/0.3M NaCl. The flow rate was 0.5 ml/min and fraction size was 1 ml. Samples were diluted 10-fold for BCDC assay. The horizontal bar indicates fractions that were pooled. (—), absorbance profile; (O----O), BCDC activity; (····), conductivity profile.

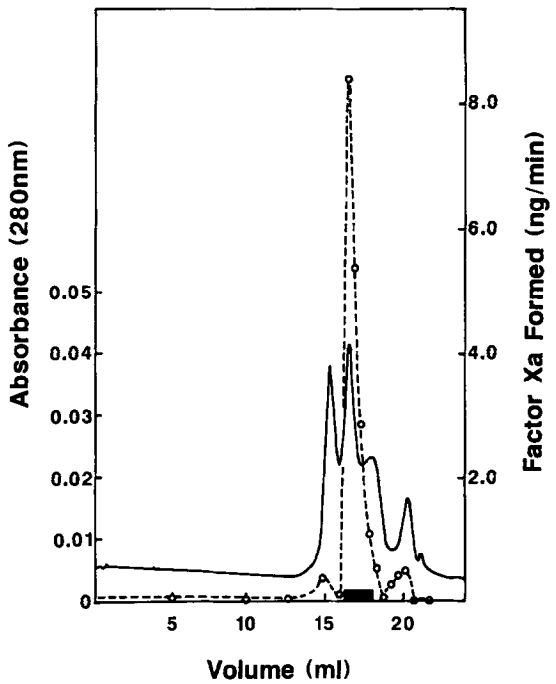
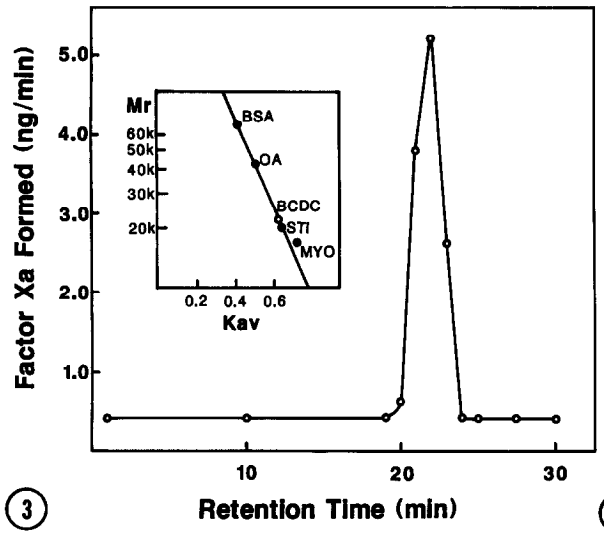


Figure 2: Elution pattern of MONO-Q purified BCDC on a Superose 6 column (FPLC). MONO-Q-purified BCDC was applied to a Superose 6 column (HR 10/30) equilibrated with 0.05M Tris-HCl (pH 7.5)/0.1M NaCl. The flow rate was 0.3 ml/min and 0.5 ml fractions were collected. Samples were diluted 10-fold for BCDC assay. The horizontal bar indicates fractions that were pooled. (—), absorbance profile; (O----O), BCDC activity.

one large activity peak eluting at 0.09M NaCl followed by a second smaller activity peak eluting at 0.12M NaCl (Figure 1).

The first MONO-Q activity peak (0.09M NaCl) was subjected to gel permeation FPLC in a Superose 6 column equilibrated with 0.05M Tris-HCl (pH 7.5)/0.1M NaCl. MONO-Q BCDC resolved into four protein peaks by Superose 6 FPLC with the BCDC activity coinciding with the second peak (Figure 2). The molecular weight of the BCDC was estimated as 25,000 and 22,000 in calibrated columns of Superose 6 and TSK-250, respectively (Figure 3). In addition, the second peak of cytokine activity eluting from MONO-Q (0.12M NaCl) also exhibited a Mr value of 22-25,000 in a calibrated Superose 6 column (data not shown).

SDS-PAGE of the Superose 6-purified BCDC revealed several minor bands and two major bands at Mr 22,000 and 12,000 (Fig. 4). Presumably, the Mr 22,000 band represents the BCDC based on the calibrated gel permeation column chromatography data presented above. We are currently raising murine monoclonal antibodies against the Mr 22,000 protein eluted from SDS-gels in an effort to (1) demonstrate specific neutralization of BCDC with this McAb, and (2) if so, assist us in the large-scale immuno-affinity purification of BCDC for more comprehensive characterization studies. In addition,



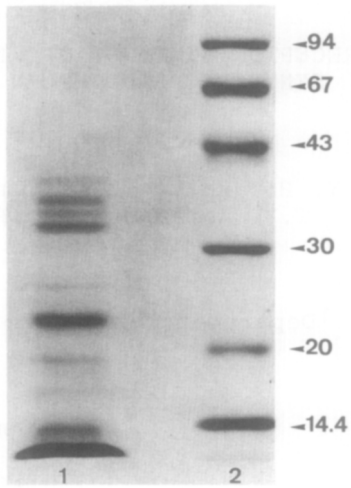


Figure 3: Bioactivity profile of Superose 6-purified BCDC on a calibrated gel permeation TSK 250 column. Superose 6-purified BCDC (250 μl) was injected onto the column equilibrated with 0.05M Tris-HCl (pH 7.5)/0.1M NaCl at a flow rate of 0.6 ml/min. The *inset* shows the calibration curve with bovine serum albumin (67,000), ovalbumin (43,000), soybean trypsin inhibitor (20,100), and myoglobin (17,000) used as standards.

Figure 4: Sodium dodecylsulfate-polyacrylamide gel electrophoresis of Superose 6-purified BCDC. Sample 1, 30 μg of Superose 6-purified BCDC; Sample 2, mixture of reduced standard proteins including 10 μg each of phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and α-lactalbumin (14,400). Electrophoresis was carried out in 12.5% polyacrylamide resolving gels according to Laemmli (7).

these McAbs may be useful in elucidating the relationship between the two peaks of cytokine activity eluting from MONO-Q FPLC (Figure 1).

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