Isolation of a Membrane Bound Immunoregulatory Molecule from Metastatic Lymphoma Cells

Contributed by T. L. McDonald[1], W. Hao[2], and S. S. Joshi[2],
[1]Department of Pathology and Microbiology, and [2]Department of Anatomy, University of Nebraska Medical Center, Omaha, Nebraska 68105

Abstract

The murine liver metastatic lymphoma cell line (RAW117-H10) significantly inhibits mitogen-induced proliferation of normal spleen cells in co-culture.[1] Cell surface membrane proteins, extracted from RAW117-H10 cells with butanol, were purified and assayed for immunosuppressive activity. The purification of the protein molecules was achieved solely by preparative isoelectric focusing in the Rotofor® cell. In less than 3 hours, 90% of the immunosuppressive activity found in the original crude butanol extract was recovered in three of twenty Rotofor cell fractions, with 80% of the immunosuppressive molecules concentrated in just one fraction.

Biochemical characterization of the Rotofor fractions containing immunosuppressive properties revealed that the cell surface immunoregulatory molecule is a glycoprotein with a molecular weight of 70 kD, and an isoelectric point of 4.3.

Methods

CELL CULTURE

Lymphoma cells were grown *in vitro* in RF10 medium consisting of RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Appropriate numbers of cells were then used for the direct co-culture experiment described below, or for extraction of cell surface molecules using n-butanol.

IMMUNOSUPPRESSIVE PROPERTY OF LYMPHOMA CELLS

Normal Balb/c spleen cells were co-cultured with mitomycin-c treated highly malignant RAW117-H10 lymphoma cells or low malignant RAW117-P cells or mitomycin-c treated normal spleen cells in presence of mitogens. There was a significant inhibition of proliferation of normal spleen cells (as determined by ^{3}H-thymidine uptake) by the highly malignant RAW117-H10 cells. Neither the mitomycin-c treated normal Balb/c spleen cells nor mitomycin-c treated low malignant RAW117-P lymphoma cells induced such inhibition. Subsequently, we isolated and purified the cell surface molecules from the RAW117-H10 cells using the Rotofor isoelectric focusing cell.

EXTRACTION OF CELL SURFACE MOLECULES

Butanol extraction was by a modification of the method of Le Grue *et al.*[3] For extraction, 2 x 10^8 lymphoma cells were treated with 2.5% butanol in phosphate buffered saline (PBS) for 5 minutes at room temperature. The cell suspension was centrifuged at 1,000 g for 10 minutes. The supernatant fluid was collected and centrifuged at 105,000 g for 1 hour, and approximately 5 ml of supernatant fluid containing released cell surface molecules were collected.

TREATMENT OF EXTRACTS FOR PREPARATIVE IEF IN THE ROTOFOR CELL

Approximately 5 ml of the cell extract in PBS were dialyzed against 0.05% n-octylglucoside overnight to remove salts. The sample was diluted to 35 ml with 0.05% n-octylglucoside, and 0.87 ml (1%) Bio-Lyte® solution (pH range 3–10; 40% w/v) was added. The cell extract was then loaded into the Rotofor cell for focusing without further treatment.

RUNNING CONDITIONS

Focusing in the Rotofor cell required less than 3 hours. The initial conditions were 12 W, 500 V, and 24 mA. At equilibrium, the values were 2,000 V, 5 mA, and 10 W. The total protein load was relatively low (~100 mg) and the ampholyte solution was 1%. As a result, the resistance of the 35 ml sample was very high and the voltage increased to the limit (2,000 V) within 2 hours.

AMPHOLYTE REMOVAL

Twenty fractions were collected, their pH values were measured, and 2.5 M NaCl was added to each fraction so that the final concentration of salt in each was 0.5 M. Fractions were then dialyzed overnight against two changes of PBS.

ASSAY FOR BIOLOGICAL ACTIVITY

For assay, 5 x 10^4 normal Balb/c spleen cells were mixed with Rotofor fractions of RAW117-H10 lymphoma cell extracts. The mixtures were pulsed with 1 µCi of ^{3}H-thymidine at 52 hours to label DNA. The cells were then incubated for an additional 18 hours, harvested on glass fiber filter paper discs, and counted. As a positive-control, spleen cells were pulsed and counted as above but were not exposed to butanol-extracted material. See Figure 1. Values obtained for spleen cells incubated with Rotofor purified RAW117-H10 extracts were compared to positive control spleen cells for determination of percent inhibition of normal spleen cell proliferation. See Table 1.

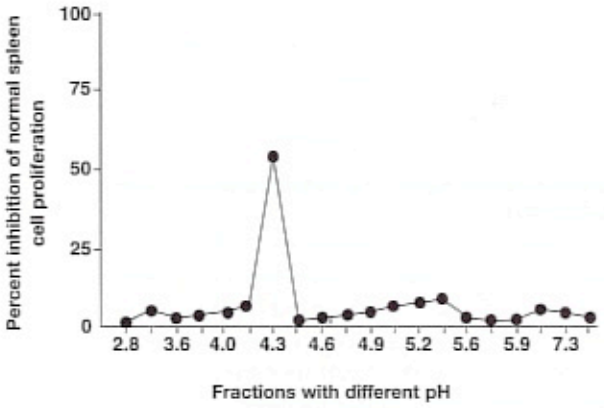

Fig. 1. Preparative isoelectric focusing analysis of immunosuppressive butanol extracts from metastatic lymphoma cells. The maximum inhibition of mitogen induced stimulation occurred (at 10 µg/ml of concentration of the molecules) with the fraction collected at pH 4.3.

SDS-PAGE ANALYSIS

Cell surface molecules from the RAW117-H10 cell crude extract were analyzed by SDS-PAGE.[4] The purified material from Rotofor cell fraction number 7, with the majority of immunosuppressive activity, was also analyzed this way. Both were compared to reference lanes containing molecular weight standards. The gels were stained with Coomassie® blue R-250 and photographed.

Table 1. Effects of Butanol Extracted Cell Surface Molecules From RAW117-Lymphoma Cells on the Mitogen Induced Proliferation of Normal Spleen Cells as Measured by Incorporation of ^{3}H-thymidine into DNA

	COUNTS PER MINUTE	
	CONCANAVALIN-A	LIPOPOLYSACCHARIDE
None (Control)	205,852±1,717	81,628±1,277
H10 Extract I*	172,884±5,542	59,331±1,718
H10 Extract II**	144,974±9,420	48,236 ±1,546[a]
H10 Extract III***	42.707±5.054[a]	29,479±4,778[a]

* *2.5 µg/well*
** *5 µg/well*
*** *10 µg/well*
a. *Significantly different from control (p ≤0.05)*

Results

The inhibition of normal spleen cell proliferation, determined by the relative incorporation of ^{3}H-thymidine into DNA, revealed that ~80% of the recovered immunosuppressive activity in material extracted with butanol from RAW117-H10 cells focused in fraction number 7 (pH 4.3), with 90% of total recovered activity focused in fractions 6–8. See Figure 1.

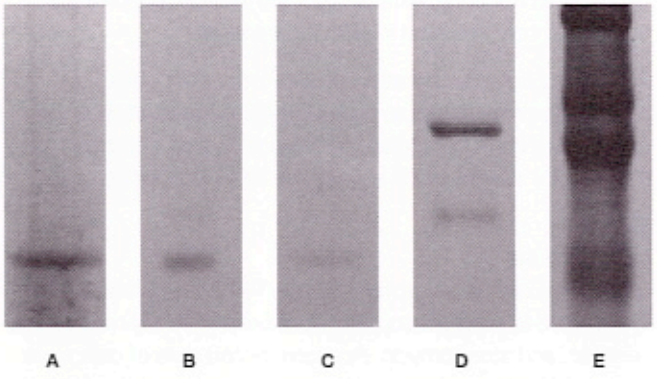

Fig. 2. Electrophoretic analysis of the Rotofor cell purified immunosuppressive molecules. The fractions that showed an inhibitory effect on mitogen induced proliferation of normal Balb/c spleen cells were analyzed by SDS-PAGE. Lane A contained crude butanol extracted molecules from RAW117-H10 cells. Lanes B and C contained different concentrations of Rotofor fraction number 7 containing the majority of immunosuppressive activity. Lanes D and E contained molecular weight markers.

SDS-PAGE analysis confirmed that the molecular weight of immunosuppressive molecules is 70 kD. See Figure 2. The 70 kD molecules could also be stained with the periodic acid Schiff reagent (PAS), confirming the glycoprotein nature of the molecules extracted from the cell surface by butanol.[5] The isoelectric point of immunosuppressive gp70 (pH 4.3) was determined by the pH of the Rotofor cell fraction that exhibited this activity. See Figure 2.

In a subsequent experiment, cell surface proteins were extracted from RAW117-H10 cells using n-octylglucoside instead of butanol. Cells were suspended in 0.05% n-octylglucoside in PBS for 15–20 minutes, then the procedure for the butanol extraction was followed. It was discovered that purification of this extracted material in the Rotofor cell did not require added carrier ampholytes. Proteins of interest were located at the same pH as when they were separated in the presence of carrier ampholytes.

Conclusion

Tumor-cell-induced host immunosuppression might be one of several mechanisms contributing to tumor progression and metastasis of highly malignant RAW117-H10 lymphoma cells. The exact mechanism of this immunosuppression can only be characterized by identifying the molecules responsible for the immunosuppression. The high voltage achieved in the Rotofor cell using 1% carrier ampholytes brought about significant purification of a 70 kD immunosuppressive cell surface glycoprotein in less than 3 hours.

The finding that material extracted from cell membranes using butanol or n-octylglucoside could be electrofocused in the Rotofor cell without the addition of carrier ampholytes is an added advantage for cell culture work. Fractions are simply assayed for activity after a short dialysis to remove detergent without having to manipulate the sample to remove ampholytes. When ampholytes were not used, the voltage in the Rotofor cell increased to 2,000 V within 1 hour and stabilized, thereby minimizing the run time.

References

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