Vol. 140, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1986 Pages 365-371

PURIFICATION OF A HEMOLYTIC FACTOR FROM RAS ONCOGENE TRANSFORMED FIBROBLASTS[1]

Janine Wieman*, Stanley Zucker[2], Dean Wilkie, and Rita M. Lysik

* Departments of Research and Medicine, Veterans Administration Medical Center, Northport, N. Y., 11768 and State University of New York at Stony Brook, Stony Brook, N. Y., 11794

Received September 2, 1986

SUMMARY -Transformation of NIH-3T3 fibroblasts by the Harvey murine sarcoma viral oncogene or by cultivation of fibroblasts under low serum conditions (spontaneous) resulted in the acquisition of hemolytic activity, as demonstrated by coincubation of the transformed fibroblasts with ^{59}Fe-labeled red blood cells. The tumor Hemolytic Factor was partially purified from conditioned media produced by T-24 human bladder transformed fibroblasts by ammonium sulfate precipitation, followed by anion and gel filtration chromatography. The hemolytic factor has a molecular weight of 66,000 as documented by SDS-PAGE and is destroyed by heating to 60°C.

The mechanism by which malignant cells damage normal host cells during the process of cancer invasion has been the subject of considerable speculation. We developed the Tumor-Induced RBC Cytolysis assay as a model system to examine this interaction (1). More than a dozen spontaneous, viral and chemically -induced animal cancer cell lines, and cell lines derived from human tumors, have been demonstrated to have the capacity to lyse normal red blood cells and their precursors during a 1-2 day incubation period (2-7). Tumor-induced erythroid cytolysis occurs at 37°C, requires direct contact between target and effector cells, and in most cases, is a calcium requiring phenomenon. Subcellular fractionation procedures have revealed that the cytolytic factor resides primarily in the plasma membranes of cancer cells (2-4).

In the current study, we have examined the acquisition of hemolytic activity following Ras oncogene and spontaneous transformation of NIH-3T3 fibroblasts. Using the conditioned media produced by T-24 human bladder cancer transformed fibroblasts, we have been able to partially purify a protein that is capable of lysing normal RBC's. We postulate that this cytoytic protein plays a role in the invasive process of cancer.

MATERIALS AND METHODS

Animals- Female C57BL/6 mice were obtained from the NCI-Frederick Cancer Facility. Wistar rats were used for the preparation of ^{59}Fe-labeled RBC's.

[1] This research was supported by Merit Review Grant funds from the Veterans Administration.

[2] To whom correspondence should be addressed at Research Building 61, V.A. Medical Center. Northport, N.Y. 11768

Abbreviations used: EDTA- ethylene diamine tetra acetic acid, Ha-MuSV - Harvey murine sarcoma virus, HEPES- (N-2-Hydroxyethylpiperazine- N'-2-ethanesulfonic acid), HF- Hemolytic Factor, MEM- minimal essential media, RBC- red blood cells, RI- Release Index, SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis, TIRC- Tumor-induced RBC Cytolysis.

0006-291X/86 $1.50

Reagents- Dulbecco's modified Eagles media and donor calf serum were obtained from Grand Island Biological Corp. ; Sigma Chemical Company supplied the chemicals used in the study; ^{59}Fe as sterile ferrous citrate was obtained from Amersham Corp.

Transformed tumor cell lines- The NIH-3T3 cell line was transformed using the calcium phosphate precipitation method with the cloned viral Harvey Ras oncogene as described by Feo, et al (8). NIH-3T3 cells were also transformed with heavy molecular weight DNA from the T24 bladder cancer cell line containing an activated C-Harvey Ras oncogene (a glycine to valine substitution at amino acid 12). (9,10). Spontaneously transformed NIH-3T3 mutants identified morphologically (more than 100 foci/flask), were grown out under low serum culture conditions, and were subcloned. Cell lines were cultured at 37°C and 5% CO_2 in flasks containing Dulbecco's modified Eagles media, 10% donor calf serum and antibiotics.

Tumor conditioned media was harvested after a 2 day incubation at 37°C of subconfluent T-24 transformed fibroblasts (10^7cells/75 mm^2 tissue culture flask) cultivated in Dulbecco's media without calf serum . Conditioned media was then centrifuged at 1500 x g for 10 min. and the supernatant was subjected to further purification at 4°C for isolation of a hemolytic factor. Solid ammonium sulfate was added slowly to the crude fraction to achieve a saturation of 60% with constant mixing and after one hour the precipitate was removed by centrifugation. Additional ammonium sulfate was added to achieve a final concentration of 100% and the final precipitate was isolated by centrifugation, redissolved in HEPES (10 mM) buffered saline (50mM), exhaustively dialysed, and then applied to a 50 x 5 mm Mono Q HR5/5 strong anion exchange column operate at a flow rate of 1 ml/min. on a Fast Protein Liquid Chromatography apparatus (Pharmacia). Following application of the sample and return of the optical density to the base line value, a 45 ml gradient of 0 to 1.0 M NaCl in HEPES buffer was used to elute the bound proteins. Fractions rich in hemolytic activity were combined, dialyzed against 1M NaCl buffer, concentrated by ultrafiltration using an Amicon YM-10 membrane, applied to a 89 x 1.6 cm Ultragel AcA 44 gel filtration column (LKB Instruments), and eluted at a flow rate of 15 ml/min. Apparent molecular weights were estimated using known molecular weight standards. Four ml factions were pooled, dialyzed, concentrated, sterilized by passage through a 0.2 um pore filter, and tested in the RBC lysis assay.

A cell fractionation procedure using nitrogen cavitation (300 psi x 15 min) followed by differential and then sucrose density gradient centrifugation was used to separate a plasma membrane enriched fraction from crude organelles, nuclei, and cytosol as we have previously described (3,4).

RBC Cytolysis Assay- The tumor-induced RBC Cytolysis Assay (TIRC) was performed in our standard fashion in tubes when examining soluble cytolytic factors and was modified as previously described for cells propagated as monolayers when examining various lines of mouse 3T3 fibroblasts (2-3). In brief, ^{59}Fe-labeled RBC's at a concentration of 5 x10^6/ ml, were added to tubes containing soluble factors or subconfluent cultures of fibroblasts in dishes containing Eagle's Minimal Essential Media (MEM lacking fetal calf serum) or Complete media (CMEM with fetal calf serum), respectively, and incubated , at 37°C in 5% CO_2 and 95% air. Control tubes and dishes containing buffer dialysate instead of hemolytic factor or cells were treated identically. After 2 days of incubation, the incubation media was harvested and a supernatant and pellet were separated by centrifugation at 2,000 x g for 10 minutes. Cytotoxicity was expressed as a Release Index (2-4): RI % = [(radioactivity released due to cell death in the supernate)/ (total radioactivity in supernate and pellet)] x 100. Inhibition of tumor-induced RBC lysis was calculated as follows: Inhibition of Cytolysis = [1-(RI drug-treated factor) / (RI untreated factor)] x 100.

Sodium dodecylsulfate polyacrylamide gel electrophoresis was performed by the method of Laemmli as previously described (11).

Collagenase and gelatinase assays were performed using native and heat-denatured [^{3}H-methyl]collagen as we have previously described (2,3).

RESULTS AND DISCUSSION

Transformed cell lines are currently being actively evaluated to determine differences in metastatic behavior between normal and malignant cells (12-15). This study has focused on the characterization of a cytolytic factor that we have previously described in cancer cells (1-5). Using the Tumor-induced RBC Cytolysis assay, we have shown that Harvey murine sarcoma virus transformed mouse 3T3 fibroblasts (Ha-MuSV) and spontaneously transformed fibroblasts are able to lyse normal red blood cells during a 2 day incubation in the presence (Table 1) or absence of fetal calf serum (data not shown). In contrast,

Table 1. Hemolytic Activity Of Transformed and Non-Transformed NIH -3T3 Fibroblasts

Sample	Release Index % ± SEM*
Dialysate control (no cells added)	3.5 ± 0.1
Non-transformed NIH-3T3 Fibroblasts	1.6 ± 0.1
Spontaneously transformed 3T3 Fibroblasts	33.5 ± 5.8**
3T3 Fibroblasts transformed by Ha-Mu-SV oncogene	54.0 ± 4.2**

$5x\ 10^5$ mouse fibroblasts were plated in dishes with Dulbecco's modified Eagles media and 10% donor calf serum. After 1 day, the spent media was removed and 5×10^6 ^{59}Fe-labeled rat RBC's in Complete Minimal Essential Media and 10% fetal calf serum were added. After 2 days of coincubation at 37° C in 5 % CO_2 and 95 % air, the RBC's were recovered from the dish and centrifuged at 700 x g for 10 min. ^{59}Fe released into the supernatant and in the remaining pellet was measured in a gamma counter and the Release Index was calculated as described in the text.

* Mean ± standard error of the mean of quadruplicate samples

** Significantly different from the control dialysate fluid at $p < .001$.

non-transformed 3T3 fibroblasts had no hemolytic activity. Using nitrogen cavitation, differential and sucrose density gradient centrifugation techniques, we were able to show that the hemolytic factor is enriched in the plasma membranes of ras oncogene-transformed fibroblasts 6 fold as compared to the crude 50,000 x g pellet (data not shown).

To purify the tumor hemolytic factor in a soluble form, subconfluent, adherent, human bladder cancer T-24 transformed 3T3 fibroblasts were incubated in flasks for 2 days in the absence of fetal calf serum. Two liters of conditioned media were collected and centrifuged at 2000 g x 10 min. to remove intact cells and debris. Ammonium sulfate precipitation at 60-100% saturation of the conditioned media, followed by redissolving the protein and dialysis in HEPES buffered saline, resulted in the isolation of the hemolytic factor which was not apparent in the crude conditioned media. Strong anion exchange chromatography on a Mono Q column at pH 7.2 led to the separation of the hemolytic factor in three protein peaks that were eluted between 0.2-0.6 M NaCl (Figure1). The active fractions were combined and placed on an Ultragel AcA-44 column (Figure 2). Three major protein peaks were eluted from gel filtration. Hemolytic activity was found in the leading edge of the third peak with an apparent molecular weight of approximately 66,000. Sodium dodecyl sulfate PAGE of the purified hemolytic factor confirmed that the hemolytic factor migrated as a broad protein band of 66,000 daltons (Figure 3) with no apparent change in molecular weight after reduction of the sample by 2% B-mercaptoethanol, indicating that HF is not made up of two chains linked by a disulfide bond. A 6 fold enrichment of hemolytic activity (as compared to the ammonium sulfate precipitate) was achieved by these chromatographic procedures.

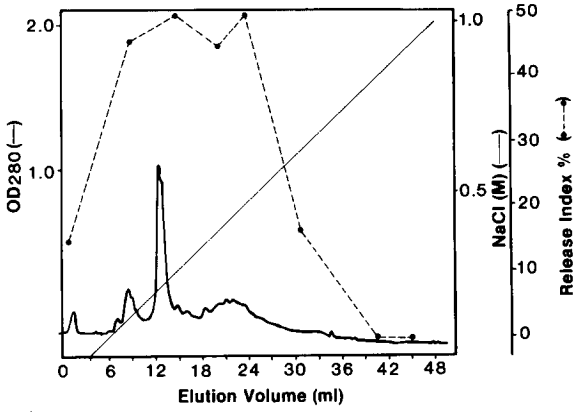

Figure 1. Anion exchange chromatography of the 60-100 % $(NH_4)_2SO_4$ precipitated T-24 transformed fibroblast conditioned media.

Precipitated proteins were dissolved in 10mM HEPES buffer, pH 7.2, and applied to a 50 x 5 mm Mono Q anion exchange column. Separation was carried out on a Fast Protein Liquid Chromatography apparatus from Pharmacia using a 45 ml gradient of 0-1 M NaCl in 10 mM HEPES. Hemolytic activity of each fraction was tested in triplicate. Symbols: Optical Density at 280 nm (——); Release Index, % (-----); NaCl, M (——).

The hemolytic factor was further characterized by showing that 57 and 92 % of the hemolytic activity was destroyed by heating the purified protein to 60°C and 90°C for one hour, respectively. Likewise, repeated freezing and thawing of the specimen resulted in complete loss of hemolytic activity. A dose-response cure for the hemolytic factor revealed that cytolytic activity was detected with a minimum of 6 ug of protein.

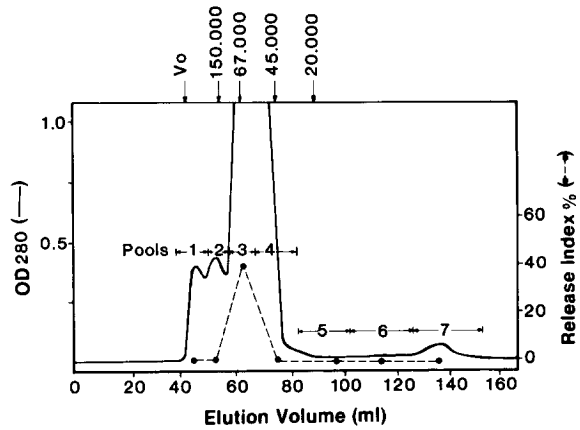

Figure 2. Ultragel AcA-44 Column Chromatography of the Active Hemolytic Fractions Obtained Following Anion Exchange.

Active fractions obtained from the Mono Q column were pooled and applied to a 1.6 x 89 cm column of Ultragel AcA-44, at a flow rate of 15 ml/hour. Five ml fractions were collected, combined into pools, and tested for hemolytic activity. Proteins used for thae calibration of molecular weights were: blue dextran, V_0, alcohol dehydrogenase (150,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (45,000 daltons), and soybean trypsin inhibitor (20,100 daltons). Symbols: Optical density 280 nm (——); Release Index, % (-----).

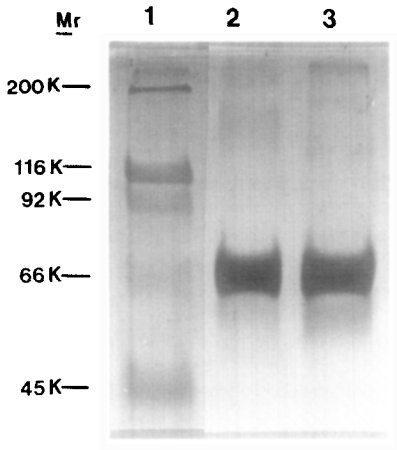

Figure 3. Sodium Dodecyl Sulfate Polyacrlamide Gel (12.5%) Electrophoresis of Partially Purified Hemolytic Factor.

Enzyme purification was performed as described in the text. Lane 1 contains the molecular weight standard proteins: myosin (200,000 daltons), B galactosidase (116,000 daltons), phosphorylase B (92,000 daltons), bovine serum albumin (66,200 daltons), and ovalbumin (45,000 daltons). Lanes 2 and 3 contain partially purified tumor Hemolytic Factor under non-reduced and reduced conditions, respectively. The gel was stained with Coomassie blue.

To determine whether tumor Hemolytic Factor had serine or cysteine proteinase activity, HF was treated with various proteinase inhibitors and then tested for RBC lytic activity. Diisopropylflorphosphate (1mM final concentration), a broad spectrum inhibitor of serine proteinases, leupeptin (0.1mM), an inhibitor of both serine and cysteine proteinases (2-4), and L-trans epoxysuccinylleucyl -amido (4-guanido)butane, a potent inhibitor of cysteine proteinases (16), had no inhibitory effect on RBC lytic activity. By contrast, EDTA (1mM), a metal chelator, inhibited HF by more than 60% and human serum diluted 1:10 completely abolished the hemolytic activity. Although Hemolytic Factor had metalloproteinase activity as demonstrated in collagenase and gelatinase assays (data not shown), which was likewise inhibited by EDTA and serum, this metalloproteinase activity was not progressively increased during the chromatographic procedures and persisted unchanged after one month of storage at 4°C, whereas the hemolytic activity was totally lost under these conditions (data not shown). These results suggest that the proteinase activity of Hemolytic Factor is due to contamination by tumor collagenase which has a similar molecular weight (17). Another potential explanation for the isolation of a Hemolytic Factor with a molecular weight of 66,000 is that a smaller active molecule might be bound to the serum albumin that tends to adhere to the surface of cultivated cells. This possibility is considered unlikely because a small molecule would have been lost in the dialysate or separated during gel filtration chromatography at 1 M NaCl.

Pozzatti et al. and Bondy et al. have presented evidence supporting the concept that the c-Ha-ras gene, as cloned from the T24 bladder cancer cell line, can confer experimental metastatic ability on NIH-3T3 cells and on primary rat embryo cells (12,13) as well as produce the morphological transformation of immortalized NIH-3T3 cells . Other investigators, however, suggest that the transformation to a metastatic phenotype appears to be a more complicated, multistep process, probably requiring the participation of more than one oncogene (14,15).

The role that the tumor Hemolytic Factor plays in cancer invasion and metastasis remains to be clarified. The observation that nonmetastatic spontaneously transformed 3T3 fibroblasts (13) have hemolytic activity, albeit less than the cloned viral Harvey Ras oncogene transformed 3T3 cells, suggests that the Hemolytic Factor may not be of primary importance in the metastatic phenomenon.

In light of our demonstration that intact tumor cells release a hemolysin, it is reasonable to reconsider the previously held theory that a circulating "carcinoma toxin" may have a role in the shortened red blood cell survival and in the anemia of malignancy (18-20) . Whereas the previous demonstration of tumor hemolysins were from necrotic areas of tumors and following vigorous cell extraction procedures, we Hemolytic Factor is from viable T-24 oncogene-transformed fibroblasts. Further characterization of specifically chose a short tumor cell incubation period and have ascertained that the release of the tumor Hemolytic Factor is needed in order to better understand the pathophysiological role of this protein. Finally, it is of interest to consider the similarities in hemolytic activity, molecular weight and requirement for calcium between tumor Hemolytic Factor and the Perforin-Cytolysin complement 9 -like molecule which has recently been isolated from the granules of large cytotoxic lymphocytes (21,22).

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Michael Viola and Ms. Shiela Oravez for providing the oncogene cell lines and for helpful advice in their maintanance.

REFERENCES

1. Zucker, S. and Lysik, R.M. (1977) Nature (London) 265:736-737.
2. Zucker, S., Lysik, R.M., Wieman, J., Wilkie, D., and Lane, B. (1985) Cancer Res.45:1419-1430.
3. Zucker, S., Lysik, R.M., Ramamurthy, N.S., Golub, L.M., Wieman, J.M. and Wilkie, D.P. (1985) J.N.C.I. 75:517-525.
4. DiStefano, J.F., Beck, G., Lane, B. , and Zucker, S. (1982) Cancer Res. 42: 207-218.
5. DiStefano, J.F., Beck, G., Mehling, K., and Zucker, S. (1983) Clin. Res. 31: 403A-405A.
6. Steven, F.S., Hulley, T.P., and Griffin, M.M. (1982) Br. J. Cancer 46: 934-939.
7. DiStefano, J.F. (1986) Cancer Res. 46: 1114-1119.
8. DeFeo, D., Gonda, M.A., Young, H.A., Chang, E.H., Lowy, D.R., Scolnick, E.M., and Ellis, R.W. (1981) Proc. Nat'l Acad. Sci. 78: 328-332.
9. Reddy, E.P., Reynolds, R. K., Santos, E., and Barbacid, M. (1982) Nature (London) 300:149-152.
10. Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M., and Wigler, M. (1982) Nature (London) 300: 762-764.

11. Laemmli, U.K. (1970) Nature (London) 227: 680-685.
12. Pozzatti, R., Muschel, R., Williams, J., Padmanabhan, R., Bruce, H., Liotta, L., and Khoury, G. (1986) Science 232: 223-227.
13. Bondy, G. P., Wilson, S., and Chambers, A.F. (1985) Cancer Res. 45: 6005-6009.
14. Bernstein.S.C., and Weinberg, R.A. (1985) Proc. Natl. Acad. Sci. 82: 1726-1730.
15. Van Roy, F.M., Messiaen, L., Liebaut, G., Gao, J., Dragonetto, H., Fiers, W.C., and Mareel, M.M. Cancer Res. 46: 4787-4795.
16. Zucker, S., Buttle, D.J., Nicklin, J.H., and Barrett, A., J. (1985) Biochim. Biophys. Acta 828: 196-204.
17. Salo, T., Liotta, L.M., and Tryggvason, K. (1983) J. Biol. Chem. 259: 3058-3063.
18. Zucker, S., Lysik, R. M., and DiStefano, J.F. (1980) J. Lab. Clin. Med. 96: 770-782.
19. Gross, L. (1947) Proc. Soc. Exptl. Biol. Med. 65 : 292-293.
20. Price, V.E. and Greenfield, R.E. (1958) Advances in Cancer Research 5: 199-290.
21. Henkart, P. (1985) Ann.Rev. Immunol. 3: 31-58.
22. Young, J.D-E, Cohn, Z.A., and Podack, E.R. (1986) 233: 184-190.