Purification and characterisation of soluble tumour haemolytic factor isolated from oncogene transformed fibroblasts

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Summary Numerous studies have shown that intact cancer cells and cell extracts have the capacity to lyse erythrocytes in vitro. The transformation of NIH-3T3 fibroblasts by ras oncogenes has recently been demonstrated to result in tumour cells releasing a haemolytic activity. Further characterisation of the soluble tumour haemolytic factor (sTHF) produced by mouse fibroblasts transformed by T24 human bladder cancer DNA and by the cloned Harvey murine sarcoma viral oncogene. To this end, transformed fibroblasts were cultivated in serum-free medium. The cell-free supernatant was treated with ammonium sulphate and the precipitate achieved at 60-100% saturation was dialysed and applied to a gel filtration column. A haemolytic factor was eluted with an M, between 65,000 and 75,000. Zinc chelate and strong anion exchange column chromatography resulted in greater than 3,000-fold enrichment of sTHF. SDS-PAGE of sTHF resulted in a single protein band of 66,000 Da. Soluble THF had no immunological cross-reactivity with known cytokines produced by lymphocytes and macrophages. The pathophysiological role of sTHF in cancer remains to be determined.

Since the turn of the century, scientists have been searching for factors, unique to tumour cells, which may be important in damaging normal cells and tissues. The anaemia of cancer, a common complication of advanced malignancy, was attributed to the elaboration of a toxin by the tumour (Weil, 1907). Supportive evidence for this hypothesis came from the demonstration that crude tumour homogenates were able to lyse red blood cells (Panzacchi, 1902; Micheli & Donati, 1903). Weil (1907) reported that the haemolytic principle of necrotic tumour extracts was dialysable whereas that of non-necrotic tumours was not.

Renewed interest in this phenomenon occurred when it was shown that cancer cells propagated in vitro were able to lyse erythrocytes (Zucker & Lysik, 1977). In some tumour cell lines the haemolytic activity was caused by a serine protease (DiStefano et al., 1982; Steven et al., 1982), but in most other cell lines tumour-induced haemolysis was a metal dependent process (Zucker et al., 1985a,b). Subcellular fractionation procedures revealed that the plasma membranes of cancer cells were considerably enriched in haemolytic activity. Purification of tumour membrane-bound haemolytic factor has been hampered by the requirement for detergents to extract the factor and maintain solubility during purification (Zucker, unpublished data).

Using transformed cell lines as a model system to analyse characteristics of cancer cells, Wieman et al. (1986) demonstrated that the transformation of NIH-3T3 fibroblasts by the Harvey ras murine sarcoma viral oncogene resulted in the acquisition of haemolytic activity by the transformed cells. A haemolytic factor was partially purified from serum-free conditioned media of bladder cancer transformed fibroblasts and was demonstrated to be a metal dependent, heat-labile protein of approximately 66,000 Da. This report describes the further purification and characterisation of this soluble THF (sTHF) isolated from both viral and human T-24 bladder cancer-transformed cells.

Materials and methods

Animals and reagents

Wistar rats were used for the preparation of 59Fe-labelled red blood cells. Chemicals were obtained from Sigma Chemicals.

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NaCl, sodium acetate buffer pH 4.5, and EDTA, pH 4.0, as described by Cawston and Murphy (1981). These purification procedures were done at 4°C. Samples were immediately neutralised to pH 7.0, dialysed against HEPES buffered NaCl (150 mM), and tested for haemolytic activity. The zinc column fraction with the highest specific activity was then applied to a 50 × 5 mm Mono Q HR 5/5 strong anion exchange column operated at a flow rate of 1 ml min⁻¹ on a Fast Protein Liquid Chromatography apparatus (Pharmacia). Following application of the sample and return of the optical density (280 nm) to the baseline value, a 42 ml gradient of 0–0.5 M NaCl in 10 mM HEPES followed by a steeper gradient to 1 M NaCl was used to elute the bound proteins. Active gel filtration fractions were also applied to a Mono P chromatofocusing column (Pharmacia) equilibrated with 0.25 M Bis-Tris, pH 7.1. Bound proteins were eluted with Polybuffer 74, pH 4.0.

To determine whether THF is secreted by the cell or released as a component of shed membrane vesicles, tumour conditioned media were centrifuged at 100,000 g for 1 h. Haemolytic activity was assayed in the pellet and supernatant.

To assess the potential haemolytic activity of residual cell-bound bovine serum albumin which is released by cells into serum-free media, the 2 day conditioned media of transformed fibroblasts was chromatographed on a Blue Sepharose CL-6B (Pharmacia) column equilibrated in 20 mM HEPES (pH 7.5) buffer containing 5 mM CaCl₂ and 0.1 M NaCl (Travis et al., 1976). Following collection of the void volume, a 1.5 M NaCl buffer was used to elute the crude albumin peak. Fractions were pooled, dialysed and tested for haemolytic activity.

**SDS PAGE electrophoresis**

A discontinuous system for polyacrylamide gel electrophoresis in sodium dodecyl sulphate was employed using the gel buffer and sample preparation system of Laemmli (1970). Gels were stained with Coomassie blue. Molecular weight standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of the known proteins.

**RBC cytolysis assay**

The tumour induced RBC cytolysis assay (TIRC) and inhibitor assays were performed as previously described (Zucker et al., 1985a; Wieman et al., 1986). Cytotoxicity was expressed as a release index (RI%):

\[
\text{RI} = \frac{(\text{radioactivity in the supernate}) - (\text{radioactivity in the supernate and pellet})}{(\text{radioactivity in the supernate and pellet})} \times 100
\]

The effect of inhibitors on RBC cytolysis were calculated using the formula:

\[
1 - \frac{(\text{RIsup} \text{trated THF or cells}) - (\text{RIcontrol})}{(\text{RIsup} \text{trated THF or cells}) - (\text{RIcontrol})} \times 100
\]

Treatment of THF with trypsin for 1 h at 37°C was performed to ascertain whether the haemolytic factor was susceptible to protease digestion. Trypsin was then inactivated with an excess of soybean trypsin inhibitor.

**Protease assays**

Collagenase and gelatinase assays were performed using native or heat-denatured 1H-methyl collagen (2 µg substrate per assay) as previously described (Zucker et al., 1985b).

**Immunological procedures**

Polyclonal antibodies to THF were produced in rabbits by a total of six injections of 360 µg of THF over a 5-month period; two subcutaneous injections of emulsified THF in complete Freund's adjuvant were followed by four intravenous injections. Rabbits were bled and IgG was isolated from serum using a Protein A-Sepharose CL-4B column as per manufacturer's instructions (Pharmacia).

In view of potential contamination of the THF preparation with bovine serum albumin (BSA), the anti THF IgG was passed through an Affi-Gel 10 column to which BSA had been covalently coupled at pH 4.8 (BioRad, Richmond, CA, USA). The unbound IgG pool was free of immunological reactivity with BSA as determined by Western blotting.

Immunoblotting was performed following transfer of proteins from an SDS-PAGE gel to nitrocellulose paper. Protein bands were probed using rabbit anti-THF IgG (diluted 1:100) and goat antirabbit IgG labelled with horseradish peroxidase as described by Spinucci et al. (1988). Immunoblotting for murine tumour necrosis factor and for lymphocyte Perforin was performed using specific rabbit polyclonal antibodies (diluted 1:100).

**Miscellaneous**

Protein determinations were made by the method of Bohlen et al. (1973) using bovine serum albumin standards.

Monoacylglycerol lipase, diacylglycerol lipase, lysophospholipase, and phospholipase C activities were determined by the method of Farooqui et al. (1984) using rac-1-S-decanoyl-1-mercaptop-2,3-propanediol, rac 1,2-S,0-didecanoyl-1-mercaptop-2, 3-propanediol, 2-hexadecanoylthio-1-ethyl-phosphocholine and rac-1-S-phosphocholine-2,3-dioctanoyl-1-mercaptop-2,3-propanediol, respectively.

Statistical analysis was done by Student's t test.

**Results**

**Haemolytic activity of intact fibroblasts**

Incubation of non-transformed 3T3 fibroblasts with ³²P-labelled RBCs did not result in lysis of the target cells, but in fact, led to a decrease in baseline RBC lysis (release index = 5 ± 2%; buffer control RI = 9 ± 1%) which appears to be related to a protective effect of the adherent 3T3 cells covering the surface of the dish. The protective effect of non-transformed 3T3 cells was not evident in non-confluent dishes. Harvey murine sarcoma viral oncogene transformed mouse 3T3 cells extensively lysed the target RBCs during a 2 day incubation period (RI = 81 ± 3%). Tumour cell induced haemolysis could be demonstrated in the presence or absence of serum in the media. In contrast, 3T3 fibroblasts transformed by T24 human bladder cancer DNA did not consistently lyse co-cultured RBCs (RI = 5–20%).

Cell doubling times of 3T3 fibroblasts, viral transformed fibroblasts, and T24 transformed fibroblasts were 23 ± 1 h, 23 ± 1 h, and 22 ± 1 h respectively in dishes containing calf serum.

**Purification and characterisation of tumour haemolytic factor**

In contrast to the intact cells, haemolytic activity was more readily purified from the conditioned medium of T24 transformed fibroblasts than viral oncogene transformed fibroblasts.

Crude conditioned medium harvested from T24 bladder transformed fibroblasts contained relatively low levels of RBC lytic activity: 0.2%, 0.3%, 0.3% and 0.4% per mg protein (results of four separate experiments). Following centrifugation of conditioned medium at 100,000 g for 1 h, the total amount of haemolytic activity was recovered in the supernatant; the resuspended pellet lacked activity. This indicates that the haemolytic factor is soluble (sTHF) and is not a component of shed vesicles.

The purification scheme that we previously reported for soluble THF with T-24 transformed fibroblast conditioned medium resulted in a 6-fold enrichment of haemolytic activity compared to the ammonium sulphate precipitated material (Wieman et al., 1986). This scheme has been modified in this report to provide more than a 230-fold enrichment in
haemolytic activity compared to the ammonium sulphate precipitated. Following ammonium sulphate precipitation (60–100% saturation), gel filtration on AcA 44 resulted in elution of haemolytic factor with an apparent molecular weight of 65-75 kDa (data not shown). Gelatinolytic activity, which was more highly enriched in the 0–60% ammonium sulphate precipitate, eluted in a fraction of lower molecular weight (Table I). Minimal collagenolytic activity was detected in any of the THF-enriched fractions. Zinc chelate column chromatography resulted in further enrichment of THF with elution of highest specific haemolytic activity with sodium acetate buffer, pH 4.5 (Figure 1). Other fractions also contained haemolytic activity, but were less pure as visualised by SDS PAGE. Anion exchange chromatography on Mono Q resulted in binding of the haemolytic activity to the column and elution at a NaCl concentration of approximately 150 mM (Figure 2a). The enrichment in haemolytic activity after anion exchange chromatography was approximately 3,800-fold compared to the starting conditioned media (Table I). Following the chromatographic purification steps, the total recovery of haemolytic activity exceeded that of the starting conditioned media. Chromatofocusing of the active fraction isolated from the gel filtration column resulted in the elution of tumour haemolytic factor (THF) at a pH between 5 and 6.

The purification of THF from Ha-MuSV transformed fibroblasts conditioned media resulted in a different chromatographic pattern than observed with T24 haemolytic factor. THF isolated from Ha-MuSV transformed cells had a similar apparent molecular weight (74,000), but was less tightly bound to the zinc chelate column and did not bind to the Mono Q anion exchange column as noted with T24 haemolytic factor (Figures 1a, 2b). Likewise, the specific haemolytic activity of THF purified from Ha-MuSV transformed cells was lower than with T24 transformed cells (0.58% lysis μg⁻¹ versus 1.15% lysis μg⁻¹ protein, respectively).

In our previous report (Wieman et al., 1986), sTHF was shown to be partially inhibited by EDTA, a metal chelator, and totally inhibited by human serum. Broad spectrum inhibitors of serine proteases and cysteine proteases had no inhibitory effect on RBC lytic activity. In the current study, no inhibition of THF was noted with soybean trypsin inhibitor (24 μM), pepstatin (0.5 μM), the aspartic protease inhibitor, phosphoramidon, (1 μM), the specific metalloproteinase inhibitor and tissue inhibitor of metalloproteinases (TIMP) (4 μM). Treatment of purified sTHF with 25 and 1,000 μg ml⁻¹ of trypsin resulted in 29 and 62% inactivation of sTHF, respectively.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of sTHF from fibroblasts transformed by either human cancer or viral Ha-ras oncogene showed sTHF to have an Mr of 66,000 (Figure 3a). Silver staining of the gel or increasing the protein content per sample did not reveal contaminating protein bands on SDS-PAGE (data not shown).

In view of the potential contamination of purified sTHF by the cell bound bovine albumin in the original culture medium, transformed fibroblast conditioned media was chromatographed on a Blue Sepharose CL-6B (Pharmacia) column. All of the cytolytic activity was recovered in the void volume. The albumin peak (M₄, 66,000), eluted with 1.5 M

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**Table I** Purification of tumour haemolytic factor

| Sample | SHA (%) | SHA enrichment | Total protein | Total activity (units HA*) | Gelatinolytic activity (μg mg⁻¹) | Gelatinolytic enrichment |
|--------|---------|----------------|--------------|---------------------------|-------------------------------|--------------------------|
| T-24 conditioned media | 0.3 ± 0.6* | 1 | 5968 | 1790 | 0.08 ± 0.01 | 1.0 |
| 0–60% (NH₄)₂SO₄ | 2.7 ± 0.5 | 9 | 541 | 1461 | 0.62 ± 0.09 | 8.1 |
| 60–100% (NH₄)₂SO₄ | 4.9 ± 1.6 | 16 | 359 | 1758 | 0.04 ± 0.00 | 0.5 |
| Ultragel AcA 44 | 125 ± 10 | 417 | 59 | 7349 | 0.30 ± 0.01 | 3.9 |
| Zn-chelate Seph. | 517 ± 24 | 1873 | 29 | 14939 | 0.25 ± 0.01 | 3.2 |
| Anion exchange (Mono Q) | 1155 ± 167 | 3849 | 4.5 | 5080 | 0.18 ± 0.03 | 2.3 |

*Mean ± standard error of the mean. *Activity = activity of sample/activity of T-24 conditioned media.

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Figure 1 Zinc chelate column chromatography of the partially purified tumour haemolytic factor. a represents the chromatogram from human bladder cancer T-24 transformed fibroblasts and b represents the chromatogram from Ha-MuSV transformed fibroblasts. The active haemolytic pools from the gel filtration column were concentrated, dialysed against borate buffer, pH 8.0, and applied to an anion-exchanged Sepharose 6B chelate column charged with ZnCl₂. Following application of the sample and washing with additional borate buffer, the bound proteins (measured at OD₂₅₀) were sequentially eluted in a stepwise manner as indicated by the arrows with 25 mM sodium cacodylate buffer (pH 6.5) in 0.8 M NaCl, then sodium acetate buffer (pH 4.5), and finally EDTA (pH 4.0). Fractions were immediately neutralised to pH 7.0, dialysed against HEPES buffered saline, and assayed without delay for haemolytic activity (expressed as release index (hatched bars)). Differences in the elution of haemolytic activity (THF) were noted between T24 and Ha-MuSV proteins.
NaCl was free of haemolytic activity (data not shown).

Soluble THF (34–61 μg per sample) purified from T24 and Ha-MuSV transformed fibroblast conditioned media did not contain detectable amounts of monoacylglycerol lipase, diacylglycerol lipase, lyso phospholipase or phospholipase C.

Immunological testing of THF

Rabbit IgG anti-THF, rendered free of reactivity with BSA, demonstrated a strong band of reactivity at M₀ = 77,000 with THF on Western immunoblots (Figure 3b). THF did not cross-react in Western immunoblots with antibodies to lymphocyte Perforin or with antibodies to tumour necrosis factor (data not shown).

Discussion

In 1977, Zucker and Lysik reported that intact rat breast carcinoma cells were able to lyse erythrocytes and normal bone marrow erythroblasts during a 24 hour co-incubation period. Tumour-induced erythroid cytolysis occurred at 37°C, required direct contact between target and viable effector cells, was independent of DNA synthesis, and was mediated by integral plasma membrane proteins (DiStefano et al., 1982). Since then, more than a dozen other spontaneous, viral and chemically transformed cancer cell lines have been shown to have the capacity to lyse erythrocytes (Lysik et al., 1979; Zucker et al., 1985b; DiStefano, 1986). Non-transformed cell lines lack haemolytic activity.

To understand better the haemolytic properties of cancer cells, we have purified a soluble tumour haemolytic factor (sTHF) from serum-free media produced by two different Harvey ras oncogene transformed cell lines. Both cell lines are highly malignant and kill virtually 100% of nude mice...
within 3 weeks of transplantation. An apparent paradox, however, is noted in measuring the haemolytic activity of intact transformed cell lines compared to their release of sTHF in vitro. Intact NIH-3T3 fibroblasts transformed by DNA from T-24 bladder cancer cells are not able to lyse RBCs in vitro. Nonetheless, T-24 transformed cells release large amounts of sTHF into 2-day conditioned media. One explanation for the lack of haemolytic activity of intact T-24 transformed fibroblasts might be the long duration of propagation of this cell line in vitro (2 years) which in other tumour cell lines has led to a disappearance of haemolytic activity (Zucker et al., 1985a). In contrast, intact virai Ha-ras transformed cells readily lyse RBCs in vitro, but release sTHF of lower specific activity than T-24 transformed cells. A possible explanation is that the haemolysis induced by intact cancer cells is mediated by a tumour membrane-bound haemolytic factor rather than s-THF. We have recently been able to extract a crude haemolytic factor from the membranes of both viral and T-24 ras transformed cells. The detergent extracted haemolytic factor differs from sTHF not only in its membrane localisation, but also in its chemical and heat stability and inhibition profile with pharmacological agents (Zucker, unpublished data).

In this study, we described a greater than 3,000-fold purification of sTHF from 2 day conditioned media produced by human T24 ras transformed fibroblasts and Ha-MuSV transformed cells. Incubation of purified sTHF with 5 million RBCs for 2 days resulted in the lysis of approximately 1% of the cells per μg protein (Table I). THF is a protein of 66,000 Da (as demonstrated by SDS-PAGE), and is susceptible to digestion by trypsin. Soluble THF was identified on immunoblotting as a protein of approximately 77,000 Da. These small differences in molecular weight determinations are probably technical in nature. Purified THF is not a serum, cysteine or aspartic protease as demonstrated by the absence of inhibition by appropriate protease inhibitors. The metal chelator, EDTA, partially inhibited THF activity indicating the metal dependence of THF. However, sequential purification of THF did not lead to enrichment of metal-dependent gelatinolytic or collagenolytic activities, thus reducing the likelihood that THF is a member of the collagenase family of metalloprotease.

Furthermore, THF was not inhibited by the collagenase-gelatinase inhibitor, tissue inhibitor of metalloproteases. THF lacked mono and dicarboxylglycerol lipase, lysophospholipase or phospholipase C activity, thus ruling out this mechanism of red cell membrane disruption.

The possibility of a relationship between sTHF and cytokines produced by T lymphocytes and killer cells (Perforin) and activated monocytes (tumour necrosis factor) was explored in this report. Perforin resembles sTHF in terms of its molecular weight, heat instability, inactivation by metal chelation or serum, and requirement for cell–cell contact for activity, but differs in several important aspects (Henkart, 1985; Podack, 1986). Perforin is capable of lysing RBCs in minutes, whereas THF requires a 2 day incubation. Perforin is localised in lymphocyte granules, whereas THF is readily released by tumour cells in vitro. Antibodies to Perforin did not cross-react with sTHF on immunoblotting or dot blottting. Of interest, Perforin has been isolated from a long-term lymphocyte cell line that no longer has cytolytic capacity (Henkart, 1985) which is analogous to our purification of THF from T24 transformed fibroblasts that lack cytolytic activity.

Antibodies to murine tumour necrosis factor (Beutler et al., 1985) did not cross-react with sTHF. Tumour necrosis factor also differs from sTHF in molecular weight and biological activity.

Having purified a protein with haemolytic capacity from tumour cells, we are presented with new questions dealing with the mechanism of action of this factor and the potential role that sTHF may play in the processes of cancer invasion or in the anaemia that accompanies disseminated cancer.

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