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Release of an M_r 140,000 Glycoprotein in the Culture Media of Certain Human Sarcoma and Melanoma Cell Lines*

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Abstract—*A* 140 K *glycoprotein was detected in the culture media of human sarcoma and melanoma cell lines by labeling with several radioactive amino acid and sugar precursors, followed by sodium dodecylsulfate-polyacrylamide gel electrophoresis and fluorography. In contrast to this, in the culture media of metabolically labeled embryonic and skin fibroblasts this glycoprotein was not found. Likewise, a protein with an identical molecular weight of* 140 K *was also found in culture media after cell surface labeling of the neoplastic cells but not in the culture media from control cells. The* [^{35}S]*methionine-labeled* 140 K *was not split by collagenase and did not appear to be a fragment of fibronectin. We discuss the possibility that secretion of the* 140 K *glycoprotein is a transformation-related phenomenon.*

INTRODUCTION

CELLS growing *in vitro* release into culture media a number of different macromolecules. Selective changes in the expression of some of these macromolecules may occur in the process of cell transformation [1–3]. For instance, many transformed cells release increased amounts of the serine protease plasminogen activator [4, 5]. On the other hand, collagen and fibronectin macromolecules responsive to cell adhesion and spreading on solid substrata are often synthesized in reduced amounts by transformed cells [6, 7]. The decreased expression of these matrix components may be related to the capacity of tumors to form metastases [8]. Moreover, neoplastic transformation affects the secretion of a number of proteins, the function of which is not yet sufficiently known.

Recently, a new cell surface glycoprotein termed GP 140 [9] or 140 kD gp [10] has been described in detergent-resistant cytoskeleton preparations of cultured human fibroblasts. According to the authors, GP 140 is a component of a glycoprotein matrix which is located between the pericellular matrix and cytoskeleton. Summarized results suggest that GP 140 may form part of a transmembrane linkage which plays a role in the organization of cytoskeleton and its anchorage to pericellular matrix and growth substratum. It is important that viral transformation of human fibroblasts decreased the quantity of GP 140 in the detergent-insoluble matrix of the cells [9].

In this report we analyzed the proteins released from human fibroblasts and several tumor cell lines. We found that some human sarcoma and melanoma cell lines released into the culture media a glycoprotein of M_r 140,000 (140 K).

MATERIALS AND METHODS

Cell cultures

Cell lines used in this study are listed in Table 1. The cells were grown at 36.5°C in Eagle's minimal essential medium (MEM) supplemented with non-essential amino acids, glutamine, 5% fetal calf serum (FCS) and antibiotics. The medium was changed routinely twice a week.

Metabolic labeling

The cultures were split 36 hr before labeling. Medium was changed immediately before labeling to Eagle's basal medium containing 5% FCS in

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Table 1. Quantification of the M_r 140 K glycoprotein in the culture media of normal and malignant human cells

Cell line	Reference	Origin	Percentage of the 140 K in the culture medium
U-20S	[28]	osteogenic sarcoma	6.2
U-3930S	[28]	osteogenic sarcoma	4.1
U-4SS	[28]	synovial sarcoma	2.9
RD	[29]	rhabdomyosarcoma	1.8
B-5GT	[21]	giant cell tumor of bone	3.5
HMB-2	[30]	melanoma	10.4
MJM	[31]	melamona	2.7
B-HM8	[30]	melanoma	0.8
VÚP	[30]	melanoma	0.2
U-2S	[28]	skin fibroblasts	ND
U-393S	[28]	skin fibroblasts	ND
U-4S	[28]	skin fibroblasts	ND
B-41FB	[32]	fibroblastic cells from bone	ND
B-42FC	[32]	fibroblastic cells from cartilage	ND
B-HEF-4	-	fibroblastic cells from whole embryo	ND
B-25F	[33]	fibroma	ND

ND = not detected.

which the concentration of glucose or methionine was lowered 10-fold. To this particular medium [^{3}H]glucosamine hydrochloride (50 μCi/ml of sp. act. 30 Ci/mmol) or [^{35}S]methionine (20 μCi/ml of sp. act. 1200 Ci/mmol; both isotopes from Amersham, U.K.) was added and labeling took place during growth at 36.5°C for 18–20 hr. Labeling with [^{14}C]proline (60 μci/ml of sp. act. 175 mCi/mol; ÚVVVR, Prague) was carried out for 16 hr in the complete medium. During this time the cultures reached half-confluency. The labeled cells grown on tissue culture dishes 60 × 15 mm (Falcon) were washed three times with fresh medium and incubated in serum-free medium for 18–20 hr. The conditioned medium containing radiolabeled proteins (from approximately 5×10^5 cells) was collected and centrifuged at 3000 *g* for 10 min to remove cell debris, filtered through a 0.22-μm single-use filter unit Millex-HA (Millipore), dialyzed against phosphate-buffered saline (PBS) and lyophilized. Radiolabeled proteins were estimated by precipitation with cold 10% (wt/vol) trichloroacetic acid followed by washes with ethanol and acetone. Aliquots of 5×10^4 counts/min were analyzed by gel electrophoresis as described below.

Lactoperoxidase-catalyzed radioiodination

The cultures were split 36 hr before labeling and grown to half-confluency in plastic dishes. The cells were washed three times with PBS at 36°C and iodination was performed by adding 0.5 mCi of carrier-free Na^{125}I (Amersham, U.K.) in 0.5 ml PBS and 25 μg lactoperoxidase (Calbiochem) to each culture [11]. The reaction was initiated by addition of 20 μl of 0.03% hydrogen peroxide. The cells were incubated at room temperature for 10 min and during this period 20 μl of 0.03% H_2O_2 were added five times to compensate for possible loss of H_2O_2 by cellular catalases [12]. The cells were immediately washed three times with PBS containing 10 mM KI, then with fresh medium, and incubated in serum-free medium overnight. After this labeling procedure and subsequent cultivation in serum-free medium 95% of the cells were viable as estimated by the Trypan blue exclusion test. The conditioned media were handled before gel electrophoresis as described above.

Immunoprecipitation

For immunoprecipitation, lyophilized [^{35}S]-methionine-labeled secreted proteins were dissolved in RIPA buffer (0.01 M $NaHPO_4$, pH 7.5, 0.1 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS). Aliquots of these proteins (2×10^5 counts/min) from each cell line were incubated overnight at +4°C with 2 μl of rabbit anti-human fibronectin serum (kindly provided by Dr A. Vaheri). Twenty-five microliters of 10% suspension of *Staphylococcus aureus* (Cowan strain I) were then added for 2 hr as a solid-phase precipitation agent [13]. The absorbed immune complexes were washed three times with RIPA buffer, each time being sedimented at 5000 *g* for 30 sec. Immunoprecipitated proteins were then solubilized by boiling for 3 min in sample buffer and examined by gel electrophoresis as described below.

Bacterial collagenase digestion

The [^{35}S]methionine-labeled samples of released proteins were incubated with bacterial collagenase (Sigma) in 0.02 M Tris-HCl, pH 7.4, containing 0.5M NaCl and 5mM $CaCl_2$ at 20 or 37°C for 24 hr

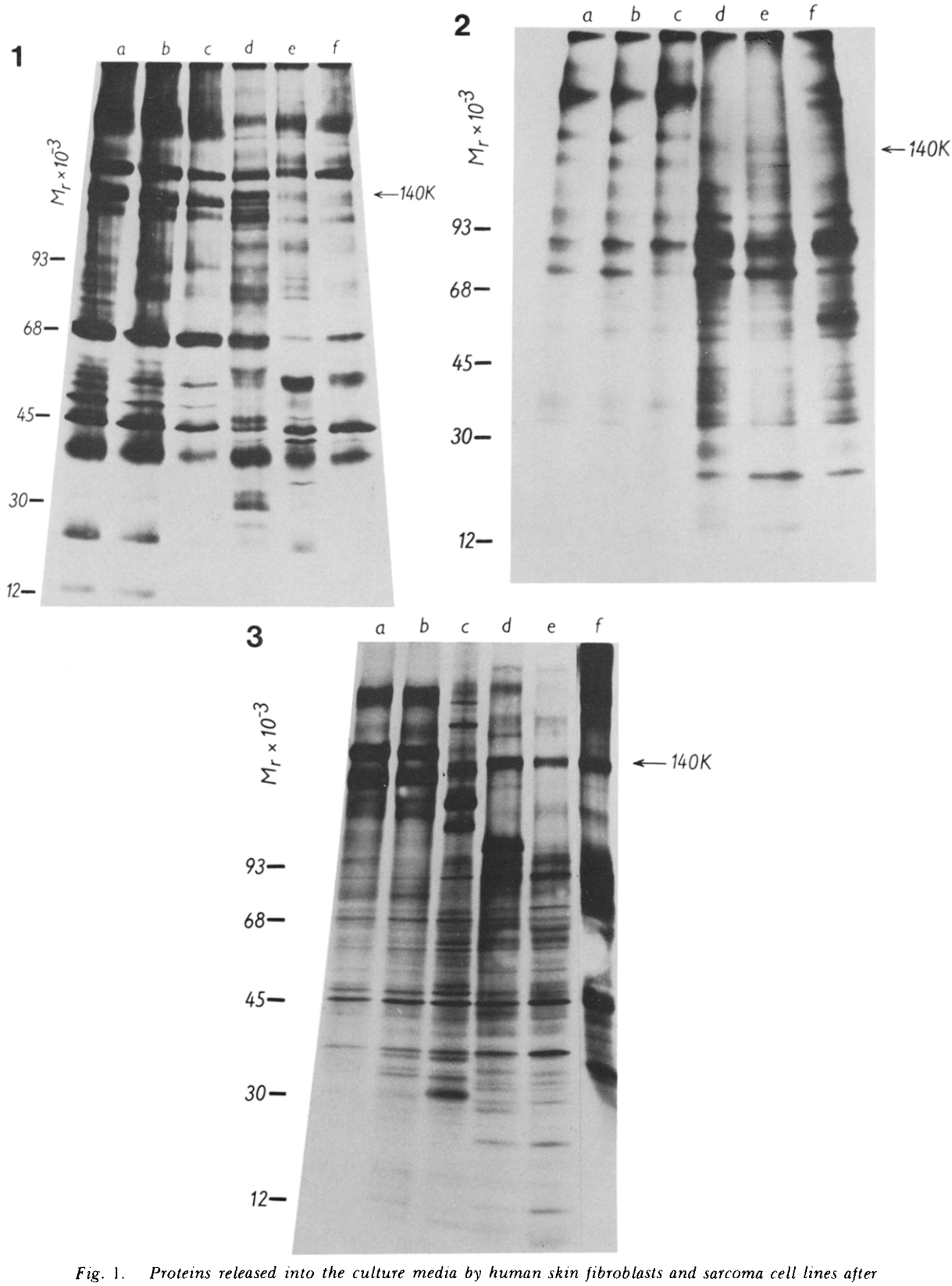

Fig. 1. Proteins released into the culture media by human skin fibroblasts and sarcoma cell lines after [^{35}S]*methionine labeling. Fluorography of the SDS-PAGE pattern. Column a, U-2S fibroblasts; column b, U-393S fibroblasts; column c, U-4S fibroblasts; column d, U-20S osteosarcoma cells; column e, U-3930S osteosarcoma cells; column f, U-4SS synovial sarcoma cells. Aliquots of serum-free conditioned media equivalent to equal radioactivity incorporated into the secreted proteins* (5×10^4 counts/min) *were analyzed. Arrow indicates the* 140 K.

Fig. 2. Polypeptide profile (fluorography of SDS-PAGE) of secreted proteins from skin fibroblasts and sarcoma cell lines after cell surface labeling with ^{125}I. *Arrangement of the samples on gel: column a, U-2S fibroblasts; column b, U-393S fibroblasts; column c, U-4S fibroblasts; column d, U-20S osteosarcoma cells; column e, U-3930S osteosarcoma cells; column f, U-4SS synovial sarcoma cells. Arrow indicates the* 140 K.

Fig. 3. SDS-PAGE analysis of the proteins released into the culture media from: column a, B-41FB fibroblasts; column b, B-42FC fibroblasts; column c, U-3930S osteosarcoma cells; column d, HMB-2 melanoma cells after [^{14}C]*proline labeling. For comparison* [^{35}S]*methionine (column e)- and* [^{3}H]*glucosamine (column f)-labeled proteins released from the melanoma cells HMB-2 were analyzed. Arrow shows the* 140 K *glycoprotein.*

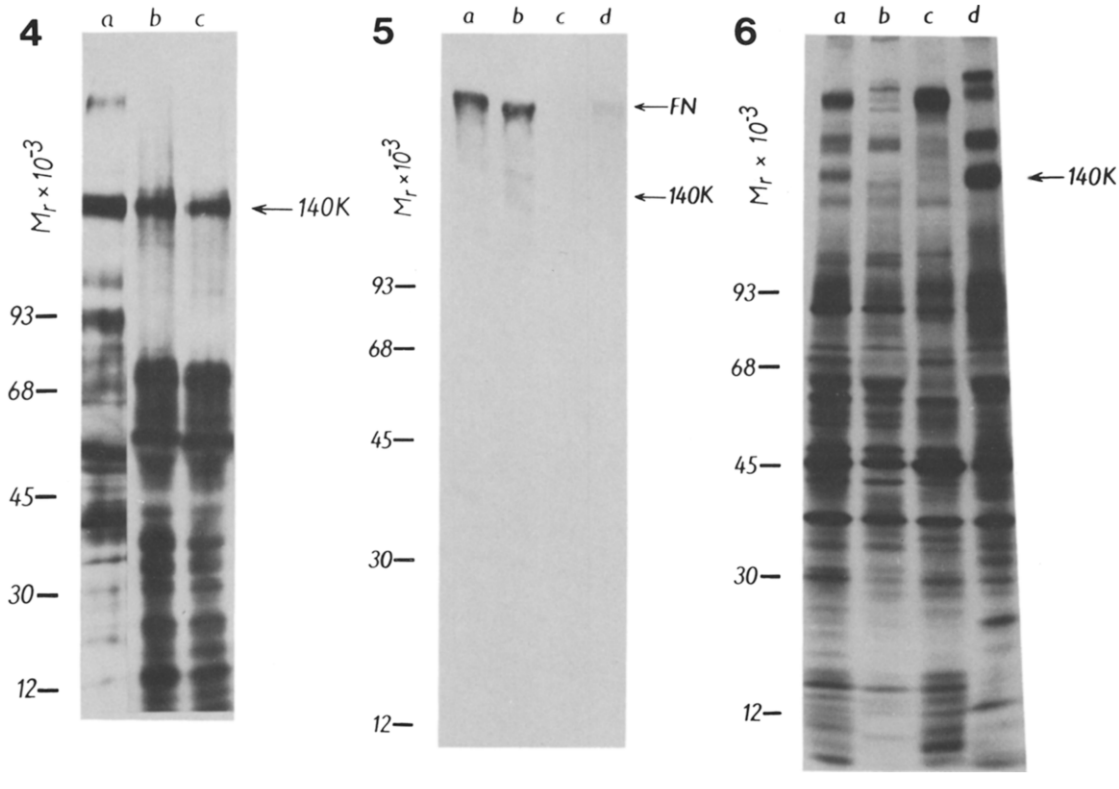

Fig. 4. Effect of bacterial collagenase on the proteins secreted by the melanoma cells HMB-2 after [35S]methionine-labeling procedure. Column a, aliquot of untreated proteins; column b, treated at 20°C; column c, treated at 37°C for 24 hr. Arrow indicates the 140 K.

Fig. 5. Immunoprecipitation of [35S]methionine-labeled secreted proteins with anti-fibronectin serum. Equal amounts of radioactivity (2 × 10⁵ counts/min) were immunoprecipitated and the total solubilized immunoprecipitate was added to one column. Autoradiogram shows the components precipitated from the individual cell lines: column a, B-42FC embryonic fibroblasts; column b, U-393S skin fibroblasts; column c, U-3930S osteosarcoma cells; column d, HMB-2 melanoma cells. The positions of fibronectin (FN) and 140 K are arrowed.

Fig. 6. SDS-PAGE analysis of [35S]methionine-labeled proteins released into the culture media from the various melanoma cell lines: column a, MJM; column 6, VÚP-1; column c, B-HM8; column d, HMB-2. Arrow indicates the 140 K.

at an enzyme:substrate ratio of 1:50. The protease inhibitors *N*-ethylmaleimide (10 mM) and phenylmethane sulphonylfluoride (2 mM) were included to eliminate non-specific proteolysis [14].

Electrophoresis and fluorography

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 1.0-mm-thick slab gels. Analyzed samples were dissolved in sample buffer (0.062 M Tris–HCl, pH 6.8, containing 5 M urea, 2.3% SDS, 10% glycerol and 5% 2-mercaptoethanol), then heated in a boiling waterbath for 3 min. Electrophoresis was performed as described by Laemmli [15] except that 5–22% gradient gels were cross-linked with *N,N'*-diallyltartardiamide. Gels were impregnated with 2,5-diphenyloxazole (PPO) as described [16], dried and placed in contact with Kodak X-Omat AR film and exposed at -75°C. Radioactivity was quantified by densitometric scanning of the developed film with an attachment to Joyce-Loebl microdensitometer. ^{14}C-Methyl-labeled standards (NEN, Boston, MA): phosphorylase B (93,000), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome *c* (12,300), were used for molecular weight estimation.

RESULTS

SDS-PAGE was used to analyze proteins released from fibroblasts and tumor cells. The [^{35}S]methionine-labeled proteins secreted by three human sarcoma cell lines and autologuos adult skin fibroblasts are shown in the fluorogram of Fig. 1. The analysis revealed differences between the proteins secreted by tumor cells and fibroblasts. Of particular note is the secretion of a 140 K protein by each of the tumor cell lines. A polypeptide of this size has been absent in the conditioned media of all normal cells so far tested (see Table 1).

A similar phenomenon was observed when the same cells were radioiodinated and subsequently cultivated in serum-free media. The labeling procedure and cultivation in serum-free media for 24 hr did not change morphology and viability of the cells, as 95% of the cells were viable as estimated by the Trypan blue exclusion test. After analysis of the proteins released into the culture media, the 140 K protein was present only in the protein spectrum of sarcoma cells (Fig. 2). The latter experiment points out probable cell surface localization of the 140 K protein.

A control experiment was performed to test whether the secretion of the 140 K protein could be related to the growth rate. No differences, however, were observed in the amount of this protein released by either a serum-starved stationary monolayer of the human melanoma cell line HMB-2 or by actively growing sparse HMB-2 cells stimulated by the addition of fetal calf serum (data not shown).

In order to test whether the 140 K protein is related to collagen, embryonic fibroblasts and tumor cells were labeled metabolically with [^{14}C]proline, which is known to label preferentially collagenous proteins [17], and the conditioned media were analyzed by SDS-PAGE. The conditioned media of B-42FC and B-HEF-4 cells (Fig. 3, columns a and b) contained several prominent [^{14}C]proline-labeled components. To compare the electrophoretic mobility of [^{14}C] proline- and [^{35}S]methionine-labeled 140 K proteins, samples of HMB-2 conditioned media were analyzed on the same gel. As seen in Fig. 3 (columns d and e), the electrophoretic mobility is identical. The 140 K was efficiently labeled by [^{14}C]proline, which is why we carried out the digestion of a [^{35}S]methionine-labeled sample of the HMB-2-secreted proteins using bacterial collagenase. In spite of the fact that several bands in distinct regions characteristic for procollagens disappeared [18], the 140 K protein seems not to be split by this enzyme at 20 or 37°C (Fig. 4, columns b and c). To fully confirm the non-collagen nature of this protein further experiments are necessary, namely the estimation of sialic acid content [17] in the purified protein and immunoprecipitations with sera against the individual types of collagens.

In the next experiments, the culture of the HMB-2 cells was also labeled with [^{3}H] glucosamine and the released proteins were analyzed by SDS-PAGE. The 140 K protein was labeled by this sugar label (Fig. 3, column f). Moreover, a protein with identical molecular weight is retained with Con-A-Sepharose (results not shown), so it is possible to suggest that the 140 K is a glycoprotein.

The possible relationship between the 140 K glycoprotein and fibronectin (subunit size M_r 220,000), known to be produced by transformed cells and to be relatively sensitive to proteolytic fragmentation [19], was ruled out by an immunoprecipitation test performed with a specific antiserum to fibronectin. This antiserum precipitates fibronectin from the culture media of fibroblasts and the melanoma cell line HMB-2 (Fig. 5, columns a, b and d). On the other hand, any band in the region of molecular weight of 220,000 is not immunoprecipitated from the culture medium of the U-3930S osteosarcoma cell line. This result is consistent with findings of Vaheri and Mosher [20], who stated that mesenchyme-derived neoplastic cells in general

fail to produce a fibronectin-containing pericellular matrix *in vitro*. None of the proteins with a molecular weight of 140,000 is precipitated from the culture media of tumor cell lines U-3930S and HMB-2. It points out the fact that fibronectin and the 140 K glycoprotein are not antigenically related.

The release of the 140 K glycoprotein was not a common phenomenon for all tumor cell lines tested (see Table 1). Quantitative differences of secretion of this glycoprotein between tumor cell lines were found. The patterns of [^{35}S]methionine-labeled proteins of the individual melanoma cell lines tested are shown in Fig. 6. According to densitometric scanning of the fluorogram, the 140 K glycoprotein constitutes about 10.4% of the proteins secreted by the HMB-2 cells (column d), 2.7% of the MJM cells (column a), 0.8% of the B-HM8 cells (column c) and 0.2% of the VÚP cells (column b) respectively.

DISCUSSION

The present results show that, besides the identifiable extracellular matrix components, some human sarcoma and melanoma cell lines release into culture media a 140 K glycoprotein of unknown function that represents a significant proportion of the macromolecules secreted by the HMB-2 and U-20S cell lines. No 140 K glycoprotein was detected in conditioned media, neither of autologous skin fibroblasts to sarcoma cell lines nor of embryonic fibroblastic cells.

Most of the cell lines used in this study have been characterized in our institute for many parameters such as morphology, growth pattern, protease activity, replicative life span, karyotype, isoenzymes and the presence of human sarcoma-associated antigens [5, 12, 21-23]. A number of differences were seen between the fibroblast cultures and tumor cell lines but some differences were also seen between individual tumor cell lines [24, 25]. We assume that differences in secretion of the 140 K glycoprotein by the tumor cell lines (see Table 1) may reflect the diversity in some of their biological characteristics which will be worth investigating in detail.

Proteins with M_r 140,000 have been described previously by several authors. It has been shown that adhesion among neural cells of chick embryo is under the developmental control of an M_r 140,000 cell surface protein [26]. Knudsen *et al.* described preparation of an antiserum against the surface membrane of transformed baby hamster kidney cells BHK/C_{13} which reversibly rounded and detached hamster cells from the substratum. Further investigation suggested that a group of glycoproteins with molecular weight around 140,000 was most likely involved in substratum adhesion and cellular morphology [27].

Recently a glycoprotein with molecular weight of 140,000 has been characterized which represented the major cell surface glycoprotein in detergent-resistant cytoskeletal preparations of cultured human fibroblasts [9, 10]. However, at the present time we do not have any direct evidence that the 140 K glycoprotein secreted by human sarcoma and melanoma cell lines is identical with the glycoproteins analyzed by Carter [9] and Lehto [10] respectively. On the other hand, some preliminary characteristics of the 140 K glycoprotein suggest this possibility, e.g. its electrophoretic mobility under reducing conditions, cell surface localization, resistance to collagenase and immunological distinction from fibronectin. Likewise, a correlation might be considered between the decreased amount of GP 140 in SV 40-transformed human fibroblasts [9] and our results describing the intensive secretion of the glycoprotein with an identical molecular weight into culture media by various types of spontaneously transformed human cells. The detergent-resistance and insensitivity of the GP 140 of human fibroblasts to a number of proteases and, on the other hand, the significant release of the 140 K glycoprotein by tumor cells permit speculation about the 140 K release from the cytoskeleton and the pericellular matrix into culture media due to interesting modifications in the molecule of this glycoprotein. A starting point to explain this phenomenon is the preparation of poly- or monoclonal antibodies against the 140 K glycoprotein. The relatively high concentration of this glycoprotein in the conditioned media of the HMB-2 and U-20S cells provides a good source for its purification.

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