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Cell Surface Response Of Chemically Transformed, Malignant Mouse Embryonal Fibroblasts And Human Colon Cancer Cells To The Maturation-Promoting Agent, N,N-Dimethylformamide (Differentiation, Fibronectin, Radioiodination).

Michael Edward Marks
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CELL SURFACE RESPONSE OF CHEMICALLY TRANSFORMED, MALIGNANT MOUSE EMBRYONAL FIBROBLASTS AND HUMAN COLON CANCER CELLS TO THE MATURATION-PROMOTING AGENT, N,N-DIMETHYLFORMAMIDE

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CELL SURFACE RESPONSE OF CHEMICALLY TRANSFORMED, MALIGNANT MOUSE EMBRYONAL FIBROBLASTS AND HUMAN COLON CANCER CELLS TO THE MATURATION-PROMOTING AGENT, N,N-DIMETHYLFORMAMIDE

by

MICHAEL E. MARKS

A DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in the
Department of Biochemistry in the Graduate School,
University of Alabama at Birmingham.

BIRMINGHAM, ALABAMA

1985

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UNIVERSITY OF ALABAMA AT BIRMINGHAM
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ABSTRACT OF DISSERTATION
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Title Cell Surface Response of Chemically Transformed, Malignant Mouse Embryonal Fibroblasts and Human Colon Cancer Cells to the Maturation-Promoting Agent, N,N-Dimethylformamide.

The lactoperoxidase/^{125}I radioiodination procedure was used to probe the cell surface of normal, nontransformed AKR-2B mouse embryo fibroblasts and malignant, permanently methylcholanthrene-transformed AKR-2B (AKR-MCA) cells to establish the relationship between cell surface changes and transformation/differentiation in this cell system. The cell surface electrophoretic profiles of AKR-2B cells indicated that the single largest amount of surface protein was a species of ∿200,000 molecular weight. AKR-MCA cells, however, had substantial amounts of 85,000 and 63,000 molecular weight surface material. AKR-2B fibroblasts transformed via application of exogenous transforming growth factors (TGF) exhibited a phenotype similar to that of AKR-MCA cells. This included a transient cell surface labeling pattern which resembled the pattern of AKR-MCA cells. The AKR-2B surface changes produced by TGF were maximally expressed between 23 and 25 hours post TGF application and were not related to mitogenesis or cell morphology.

AKR-MCA cells displayed surface alterations secondary to N,N-dimethylformamide (DMF)-promoted differentiation. Growth of AKR-MCA cells in DMF virtually eliminated the 85,000 and 63,000 molecular weight surface proteins susceptible to radioiodination and increased surface material of ∿200,000 molecular weight. Thus, surface profiles of DMF-treated AKR-MCA cells were essentially identical to those of nontransformed AKR-2B cells. AKR-MCA surface alterations resulting from growth in DMF were dependent upon time of exposure to DMF and DMF concentration. DMF also promoted AKR-MCA cell surface expression of fibronectin and prevented the morphological and cell surface changes associated with exposure of AKR-2B fibroblasts to TGF.

After demonstrating the relation between the cell surface and the state of differentiation/transformation in the AKR-2B/AKR-MCA system and illustrating the ability of DMF to induce surface modifications correlated with differentiation in this system, experimentation was extended to a cultured human colon cancer cell line (HCT MOSER). HCT MOSER cells exposed to DMF manifested marked, reversible morphological and surface changes which occurred as a function of time of growth in DMF and DMF concentration. Interestingly, material reactive with anti-fibronectin was found on the surfaces and in the culture medium of DMF-treated HCT MOSER cells.

Abstract Approved by: Committee Chairman John M. McKelvin

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LIST OF ABBREVIATIONS

AKR-MCA	methylcholanthrene-transformed AKR-2B mouse embryo fibroblasts
BSA	bovine serum albumin
BTP	1% bovine serum albumin, 0.05% Tween 20 in phosphate-buffered saline
CEA	carcinoembryonic antigen
cpm	counts per minute
csp63	radioiodinated cell surface protein with a molecular weight of 63,000
csp85	radioiodinated cell surface protein with a molecular weight of 82,000 to 87,000
csp120	radioiodinated cell surface protein of 120,000 molecular weight
csp180	radioiodinated cell surface protein of 180,000 molecular weight
csp200	radioiodinated cell surface protein with a molecular weight of 200,000 to 250,000
DMF	N,N-dimethylformamide
EGF	epidermal growth factor
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
mCi	millicurie
mol. wt.	molecular weight

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline (0.01 M sodium phosphate, 0.154 M sodium chloride, pH 7.4)
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
std. dev.	standard deviation

GENERAL INTRODUCTION

Neoplasia has been postulated to be a disease of aberrant cellular differentiation (3,49,58)*. Pierce (72) and Potter (73) have suggested that many tumors are stem cell tumors. As such, these tumors might be subject to treatment involving induction of a more benign phenotype. The ability of exogenous agents to influence differentiation was suggested as early as 1925 when Wolbach and Howe (103) reported that vitamin A deficiency in rats resulted in abnormal differentiation and proliferation of epithelial cells. Other investigators have since observed that animals deficient in retinoic acid tend to develop squamous metaplasia in the epithelia of the eye, nasal mucosa, respiratory tract, genitourinary tract, salivary glands and pancreatic ducts (7,39,41, 42,105).

Many compounds including retinoic acid have been investigated for their ability to induce a more differentiated phenotype when applied to cancer cells in vitro (9,16,17,30, 31,37,49,55,88,89). One such compound, N-methylformamide, has been evaluated for therapeutic potential in Phase I clinical trials (62,71). N-methylformamide is the product of

* References for this section begin on page 136.

N,N-dimethylformamide (DMF) metabolism in humans (32). The maturation-promoting capabilities of the polar solvent DMF have been extensively studied *in vitro*. Dexter and coworkers (30,37) have shown that human colon cancer cells grown in the presence of DMF manifest reduced tumorigenicity and clonogenicity in soft agar, exhibit increased expression of carcinoembryonic antigen (CEA) and have decreased expression of tumor-colonic mucoprotein antigen and H-gene determinant. In cultured human colon cancer cell systems DMF has been reported to enhance sensitivity to mitomycin C and cis-platinum (29), affect the activities of enzymes involved in purine metabolism (28) and increase susceptibility to ionizing radiation (31,50). Hence, DMF may prove efficacious in combination protocols.

The differentiation-inducing effects of DMF are not limited to colon cancer cells. DMF has been demonstrated to promote differentiation in Friend murine erythroleukemia cells (69), human HL-60 promyelocytic leukemia cells (23) and murine rhabdomyosarcoma cells (27). The Friend erythroleukemia system is perhaps the best characterized *in vitro* model for the study of differentiation (36,57,69,81,90,91). However, the Friend erythroleukemia cell line is a very specialized system and one which, upon exposure to maturational agents, undergoes terminal differentiation (i.e., maturation to orthochromic erythrocytes which do not revert to the undifferentiated state upon removal of the differentiation inducer) (36,81,90).

Chemically transformed fibroblast lines offer an attractive model system for the investigation of differentiation promotion because of the availability for comparison of the normal fibroblast lines from which the transformed cells are derived. One such cell line is the methylcholanthrene-transformed AKR-2B mouse embryonal fibroblast line (AKR-MCA). AKR-MCA cells are considered to be malignant based on the criteria of morphology, anchorage-independent growth and tumorigenicity in nude mice (59). However, when grown in culture medium containing DMF, AKR-MCA cells assume a phenotype similar to that of the untransformed parental AKR-2B cells (19). DMF induces malignant AKR-MCA cells to respond to normal growth controls as indicated by restoration of EGF mitogenic response, increased doubling time, loss of anchorage-independent growth and reduction in saturation density (19).

Sporn et al. (13,86) have suggested that the differentiation-induction mechanism of transformed cells may involve certain polypeptide growth factors which have been termed transforming growth factors (TGFs). TGFs are polypeptides of molecular weight 6,000 to 30,000 which are heat- and acid-stable and have the general property of inducing phenotypic transformation and anchorage-independent growth in untransformed target cells (52,59). TGFs were first discovered in conditioned medium from murine sarcoma virus transformed mouse fibroblasts and were originally designated sarcoma growth factors (24). Since then polypeptides with similar

properties have been found in other transformed cell types as well as in embryonal cells (65,74,75,93,98). The presence of TGFs in nontransformed tissues suggests that these factors may be functionally related to normal development and that inappropriate production of TGFs may lead to cellular transformation.

Moses and his colleagues have used the AKR-2B fibroblast line as target cells for the identification of TGFs (65). Moses et al. (65,97) have also demonstrated that transformed AKR-MCA cells produce and respond to TGFs.

TGFs have been identified in the conditioned medium and cell extracts of the human colon cancer cell line HCT MOSER (52). HCT MOSER is a line which has been established in vitro from a primary tumor obtained at surgery (14) and, by virtue of in vitro parameters, has been classified as a moderately differentiated, intermediately aggressive colon carcinoma line (14,60).

Cell surface proteins have been associated with the malignant biological cell properties of various cancer cell types as well as the biological functions of normal cells (5,33,44,67,95,109). The cell surface tends to reflect intracellular and environmental changes in a dynamic manner (101). According to Moscona (64) the cell surface is normally involved in control of cell differentiation and cell replication by functioning as an interface for signals between the outside to the inside of the cell. Furthermore, various extracellular factors produce changes in the cell

surface which, in turn, produce changes in intracellular processes.

In addition to the association of the cell surface membrane of nontransformed cells with such cellular processes as contact inhibition, immune response, regulation of cell proliferation and differentiation (38,76,77,80,84), the cell surface is also related to certain biological properties of cancer cells. Nicolson (66) has reviewed evidence suggesting that the cell surface is involved in metastasis and evasion of host-mediated immune destruction of tumor cells. Cell surface characteristics have been found to distinguish human colon cancer cell lines which manifest different degrees of aggressiveness *in vitro* (15,60). Baylin *et al.* (5) have shown that human small-cell and non-small-cell lung cancer can be differentiated by cell surface phenotype. Alterations in the cell surface have been demonstrated to occur concomitant with drug resistance in cancer cell systems (6,102).

Although the mechanism of action of differentiation-inducing agents has yet to be clearly elucidated, there is evidence that the cell surface membrane is involved in the differentiation process produced by certain compounds. Working with the Friend erythroleukemia cell line, Lyman *et al.* (57) observed that cells responding to dimethyl sulfoxide (DMSO)-promoted maturation exhibited decreased membrane fluidity. Tapiero *et al.* (91) showed that membrane microviscosity increased in Friend cells susceptible to differentiation produced by DMSO. Friend cells resistant to DMSO-induced

differentiation displayed no change in membrane microviscosity. Similar observations were made by Ip and Cooper (47) in their experiments with the HL-60 human promyelocytic leukemia line.

Retinoids have been hypothesized to exert their maturation-promoting actions in a manner analogous to steroid hormones, i.e., retinoids enter the nucleus after first binding to a cytoplasmic retinoid-binding protein (22,25). After entry into the nucleus, retinoids are thought to affect gene expression (21,22,25). However, Yen et al. (108) have shown that interaction of retinoic acid limited to the cell surface membrane is sufficient to elicit differentiation and G I/O-specific growth arrest of HL-60 cells. Lotan et al. (56) have proposed that the cell surface changes produced by growth of the S91 murine melanoma cell line in retinoic acid may be responsible for subsequent growth inhibition. These results suggest that alteration of the cell surface is the mechanism by which some differentiation agents act.

Most studies have used morphological or biological criteria to assess differentiation and, until recently, there has been scant regard to the molecular basis of maturation-induction or molecular changes associated with differentiation. This is particularly true for solid tumor cell lines in which maturational events are more difficult to study (87). The present study involves an examination of the response of transformed, malignant mouse fibroblasts and human colon cancer cells to DMF at the level of the cell surface.

This investigation was conducted in three phases. Phase I concerned the demonstration of cell surface changes associated with transformation of the putatively normal AKR-2B mouse embryonal fibroblast line by chemical and biological agents. Phase II documented that cell surface alterations correlate with other phenotypic parameters in indicating that permanently, chemically transformed AKR-MCA cells exposed to DMF assume the characteristics of nontransformed AKR-2B cells. Phase III determined the effects of DMF on the cell surface features of the human colon cancer cell line HCT MOSER.

The AKR-MCA cell line was used in these experiments because of the previously mentioned advantages of availability of the normal, nontransformed AKR-2B cells for comparison and the demonstrated biological responsiveness of AKR-MCA cells to DMF. Also, AKR-2B fibroblasts become transiently transformed when exposed to TGFs produced by AKR-MCA or HCT MOSER cells (52,59,61). Thus, the AKR-MCA/AKR-2B model system provides an additional advantage in the investigation of molecular events associated with differentiation induction because the opposite of differentiation, i.e., transformation, can be examined in the same system using two very different means to produce transformation.

Use of the HCT MOSER line in Phase III of this study presented a significant drawback inherent in the usage of any human colon cancer line in that there is no satisfactory normal human colon cell line established <u>in vitro</u> which

could serve as a reference. However, given that colorectal carcinoma is one of the most common malignant diseases in this country, has an annual mortality rate of approximately 60,000 persons and is a disease for which the survival and cure rates have not improved during the past two decades (48,82,83), the need for effective therapeutic measures is obvious. In this regard, Spremulli and Dexter (87) have indicated the importance of examining the response of colon carcinoma cells to maturational compounds. Furthermore, Wolman and Mastromarino (104) have commented on the need for markers of colonic cell differentiation regarding neoplastic transformation. Investigators may identify such markers by studying the molecular modifications resulting from differentiation of colon cancer cells, i.e., transformation in reverse.

This dissertation is comprised of two previously published manuscripts as well as unpublished data presented in the last section entitled "N,N-Dimethylformamide-Induced Cell Surface Modification and Enhanced Synthesis of a Fibronectin-Similar Protein in Cultured Human Colon Carcinoma Cells." References for the last section and for this introduction are listed under "References" beginning on page 136.

INDUCTION OF PLASMA MEMBRANE ALTERATIONS IN AKR-2B MOUSE EMBRYO FIBROBLASTS BY ENDOGENOUS GROWTH FACTORS FROM MALIGNANT HUMAN CELLS

by

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ABSTRACT

AKR-2B mouse fibroblasts were treated with 50 μg/ml of crude transforming growth factor (TGF) of human origin. Cell surface proteins of treated cells were radioiodinated and compared to untreated cells at various times after the addition of TGF. Treated cells showed a several-fold increase (∿6-fold) in cell surface ^{125}I incorporation relative to normal cells at 24 h. Electrophoretic comparisons of treated and untreated cells showed large increases in the labeling of cell surface proteins of mol. wt. 50,000-90,000 from TGF-exposed cells between 10 and 24 h post treatment. By 48 h post treatment, the electrophoretic profiles of TGF-exposed cells had returned to a pattern similar to that of untreated cells. However, even after a 48 h exposure to TGF, the cells retained a transformed morphology indicating that the electrophoretic alterations were not simply due to the morphological transformation induced by TGF. The electrophoretic pattern of TGF-treated cells at 24 h post treatment was similar to that of AKR-2B cells permanently transformed by treatment with methylcholanthrene, but was clearly distinct from that induced by treatment of normal AKR-2B cells with

epidermal growth factor (EGF). EGF induced an increase in a protein of mol. wt. 60,000 in the electrophoretic profiles taken 24 h post treatment. As with TGF, the appearance of electrophoretic profiles of EGF-treated cells returned to "normal" by 48 h. Again, these alterations did not appear to be dependent upon morphological changes since EGF-treated cells showed a morphological transformation similar to that of cells treated with TGF, and this was maintained throughout the 48 h experimental period.

INTRODUCTION

Endogenous growth factors have been isolated from a large variety of neoplastic and non-neoplastic cells (Todaro et al., 1980; Roberts et al., 1981, 1982a,b; Moses et al., 1981; Tucker et al., 1983; Halper and Moses, 1983; Twardzik et al., 1982; Kaplan et al., 1982). These factors, referred to as TGFs, have the general property of inducing phenotypic transformation and anchorage-independent growth in untransformed target cells. Moses and his colleagues have introduced AKR-2B embryo fibroblast cells as a target cell for the identification of TGFs (Moses et al., 1981). AKR-2B cells represent an interesting target system for TGFs since these investigators have shown that their chemically transformed counterparts (designated AKR-MCA cells) produce and respond to TGFs (Moses et al., 1981; Tucker et al., 1983). AKR-MCA cells are malignant by the criteria of morphology, anchorage-independent growth and tumorigenicity in nude mice. Other studies by this group (Moses et al., 1978) indicated that the endogenous growth factors from AKR-MCA cells reduced their requirement for exogenous factors such as epidermal growth factor (EGF).

Membrane proteins and glycoproteins have been thought to play a role in determining the biological properties of malignant cells (Nicolson et al., 1977; Marks et al., 1984; Baylin et al, 1982). The availability of the AKR-2B - AKR-MCA cell systems offered us the opportunity to determine whether there were significant differences in the membrane proteins of the two types of cells and, if such differences were observed, to determine whether TGFs could induce alterations in the membrane proteins of AKR-2B cells. Utilizing a previously described methodology for the radioiodination of plasma membrane proteins (Marks et al., 1983; Brattain et al., 1983), we found significant differences between the sodium dodecyl sulfate polyacrylamide gel electrophoretic patterns of radiolabeled proteins from malignant and non-transformed cells. Treatment of AKR-2B cells with crude TGFs from a human cell line resulted in the reversible assumption of a membrane labeling pattern similar to that exhibited by malignant cells. The labeling pattern induced by the crude TGFs was different from that induced by epidermal growth factor (EGF) in AKR-2B cells.

MATERIAL AND METHODS

Lactoperoxidase, glucose oxidase and EGF were purchased from Sigma (St. Louis, MO). Carrier-free $Na^{125}I$ at a concentration of 100 mCi/ml was obtained from Amersham (Arlington Heights, IL). All reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), including high- and low-molecular weight standards, were from Bio-Rad (Richmond, CA). Cell culture supplies were obtained from both Gibco (Grand Island, NY) and Flow Laboratories (McLean,VA) and cells were maintained in Corning disposable flasks.

TGF. TGF was isolated from the serum-free conditioned medium of the previously described cultured human colon carcinoma cell line HCT MOSER (Brattain et al., 1981, 1982). The methodology utilized for TGF isolation was described by Marquardt and Todaro (1982). Briefly, serum-free medium was clarified by centrifugation (100,000 g, 45 min), dialyzed against 1% acetic acid (v/v) and lyophilized. Due to the small amounts of material available, the resulting crude extracts of serum-free medium were utilized in the experiments described below. However, characterization of the crude extracts showed that anchorage-independent colony formation

(>20 cells/colony) by AKR-2B cells was of the order of 500 colonies/35-mm plate/25 µg protein. Exposure of the crude extract for 1 h to dithiothreitol or trypsin completely eliminated colony-forming ability. Fractionation on BioGel P10 showed peaks with colony-forming activity at ∿14,000 and 6,000 daltons. The 6,000 dalton peak was capable of competing with ^{125}I-labeled EGF for placental plasma membrane receptors (assays kindly performed by Dr. Alan Levine of the Department of Pharmacology).

Treatment of Cells with TGF. AKR-2B and AKR-MCA cells (obtained from Dr. H. L. Moses, Mayo Clinic, Rochester, MN) were maintained at 37°/5% CO_2 in McCoy's medium supplemented with 10% heat-inactivated fetal bovine serum and 52 µg/ml gentamycin. Cells were grown to confluency 24 h prior to treatment with polypeptides. TGF treatments were performed on AKR-2B cells by replacing the culture medium with fresh growth medium (5 ml) containing 50 µg/ml of lyophilized protein from the crude extracts of serum-free conditioned medium described above. EGF treatments were performed in the same manner at 10, 50 and 250 ng/ml. Control cultures received complete changes of fresh growth medium without additional growth factors. Cultured cells were labeled in situ at various times after treatment with growth factors using the methods described below. AKR-MCA cultures were radioiodinated at confluency.

Radioiodination and Electrophoresis. Procedures for radioiodination and electrophoresis were performed

essentially as previously described (Marks et al., 1983; Brattain et al., 1983). Briefly, cell cultures were washed three times with phosphate-buffered saline (0.01 M sodium phosphate, 0.154 M sodium chloride), pH 7.4 (PBS) and incubated at room temperature for 10-12 min in 3 ml of PBS containing 1.5 units of lactoperoxidase, 1.5 units of glucose oxidase, 30 mg of beta-D-glucose and 1.0 mCi of $Na^{125}I$. The reaction mixture was then removed, cells were washed three times with PBS and harvested by scraping in PBS. Radioiodinated cells were pelleted by centrifugation (800 g for 5 min) and osmotically lysed with periodic vortexing in water with 1 mM phenylmethylsulfonyl fluoride (PMSF). An aliquot of the cell lysate was removed for DNA determination according to the procedure described by Olander et al. (1982). The remainder of the cell lysate was centrifuged at 3,000 g for 10 min, and the supernatant containing cytoplasmic proteins (which should not be ^{125}I-labeled) was subjected to electrophoresis as a control for cell-surface labeling. The pellet containing ^{125}I-labeled surface components was solubilized by heating at 70^0 for 20 min in 2.5% SDS (w/v), 5% beta-mercaptoethanol (v/v) and 2 mM PMSF.

SDS-PAGE (0.1% SDS, 6% polyacrylamide) of solubilized cell surface components was performed by the method of Weber and Osborn (1969) on 5.5 X 100 mm cylindrical gels. Molecular weight standards (14-200 Kd) were run concurrently. After electrophoresis for $\sim$4 h at 10 mA/tube, the gels were removed and stained with Coomassie brilliant blue R-250 in

aqueous methanol-acetic acid. After destaining, the gels were sliced at 1-mm intervals with a Bio-Rad Model 195 electric gel slicer. The gel slices were counted directly in a Beckman Gamma 4000 gamma counter.

In addition to electrophoresis on cylindrical gels, radiolabeled cell surface material from AKR-2B cells exposed to TGF for various times was electrophoresed on 5-10% (w/v) linear acrylamide gradient slab gels in 0.1% SDS (w/v) using 4% acrylamide stacking gels. Preparation of the gel solutions and electrophoresis of the samples was based on the procedure of Weber and Osborn (1969). Molecular weight standards were run along with the labeled samples. After electrophoresis, slab gels were stained and destained as above and then dried. The regions of the gels containing radiolabel were detected by autoradiography on Kodak XAR-5 film.

RESULTS

Treatment of AKR-2B cells with 50 μg/ml of crude TGF resulted in transformation within 12 h to a morphology similar to that of permanently transformed AKR-MCA cells (Fig. 1). This morphological transformation was maintained throughout the 48 h period of these experiments. All concentrations of EGF (10-250 ng/ml) tested also induced morphological transformation of AKR-2B cells which persisted for the entire experimental period.

Typical electrophoretic patterns of AKR-2B and AKR-MCA cells are shown in Figure 2C and 2B respectively. The major lactoperoxidase-^{125}I-labeled cell surface component from AKR-2B cells was approximately 200,000 daltons. The labeling of other components was relatively insignificant compared to the 200,000 mol. wt. material. The difference between the patterns of the normal cells and permanently transformed AKR-MCA cells is striking. AKR-MCA cells showed significant peaks of labeled material with mol. wt. ∿82,000 and 63,000 in addition to the material with mol. wt. ∿200,000.

Some variability of labeling was encountered with TGF-treated cells at 24 h post treatment, but in all cases the

Figure 1 - Culture morphologies of (A) AKR-2B cells; (B) AKR-2B cells treated with TGF; and (C) AKR-2B cells chemically transformed by methylcholanthrene (AKR-MCA). AKR-2B cells in (B) were exposed to 50 µg/ml crude TGF at a sub-confluent stage and manifested the morphology shown beginning 7-10 h after application of TGF (X 65).

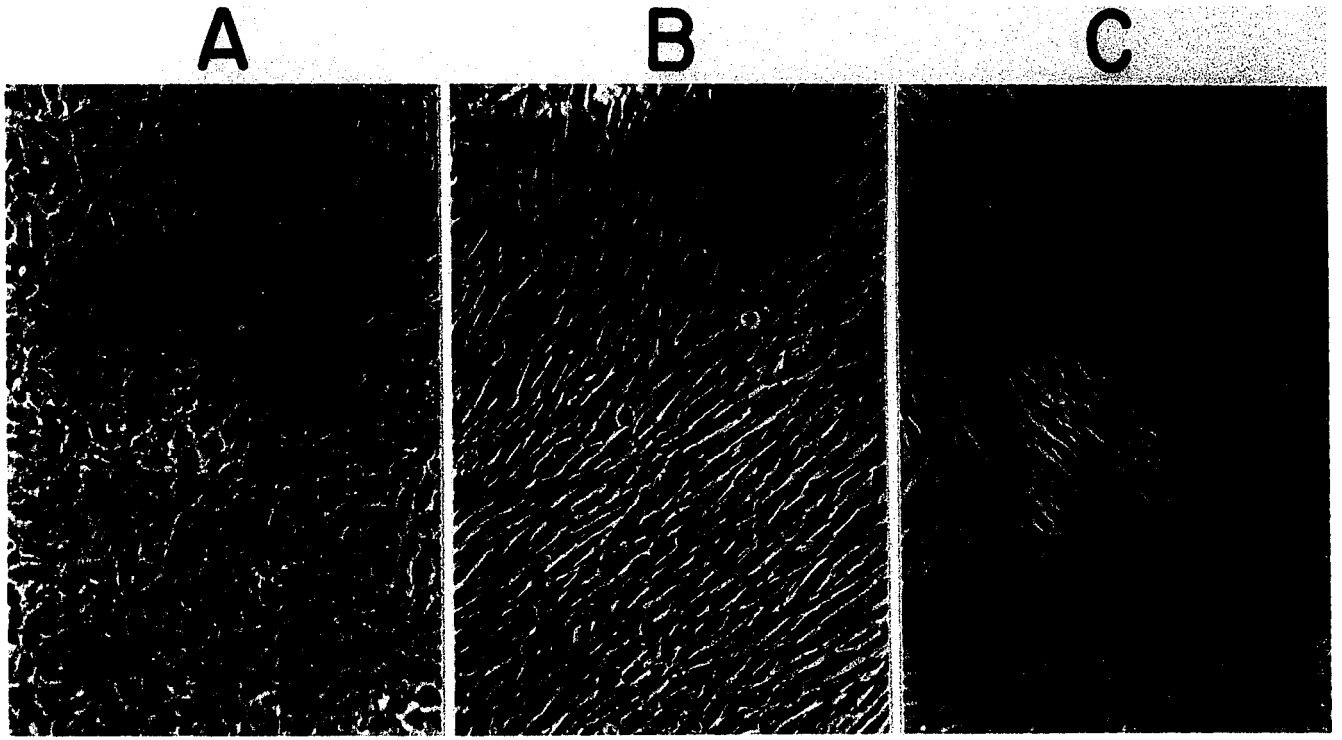
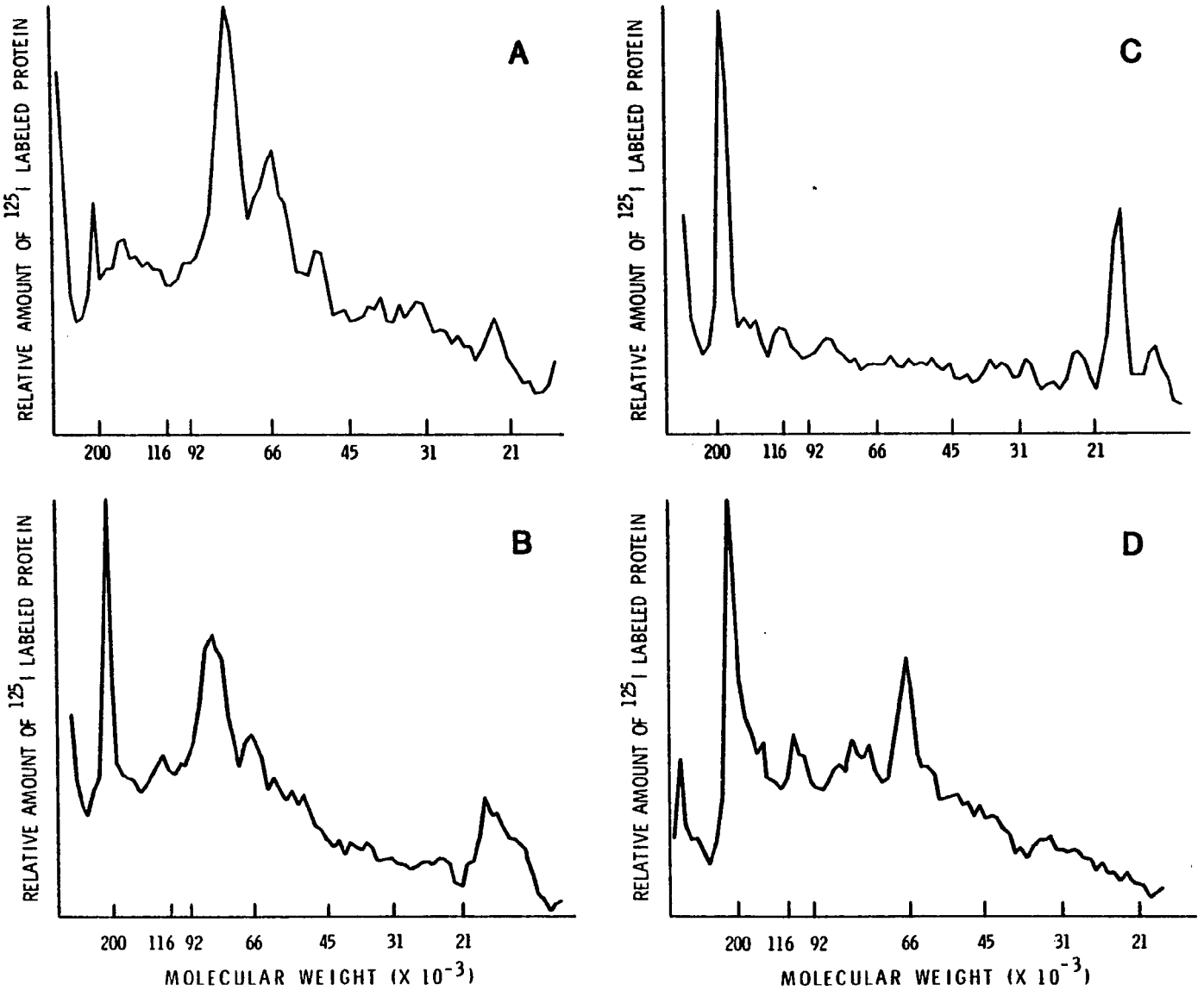

A
B
C

Figure 2 - Electrophoretic profiles of ^{125}I-labeled cell-surface proteins from: (A) AKR-2B cells 24 h after exposure to 50 μg/ml crude TGF; (B) AKR-MCA cells; (C) untreated AKR-2B cells; and (D) AKR-2B cells 24 h following exposure to 50 ng/ml EGF. The cells were grown and labeled, and the radioiodinated proteins were electrophoresed as described under "Material and Methods."


A
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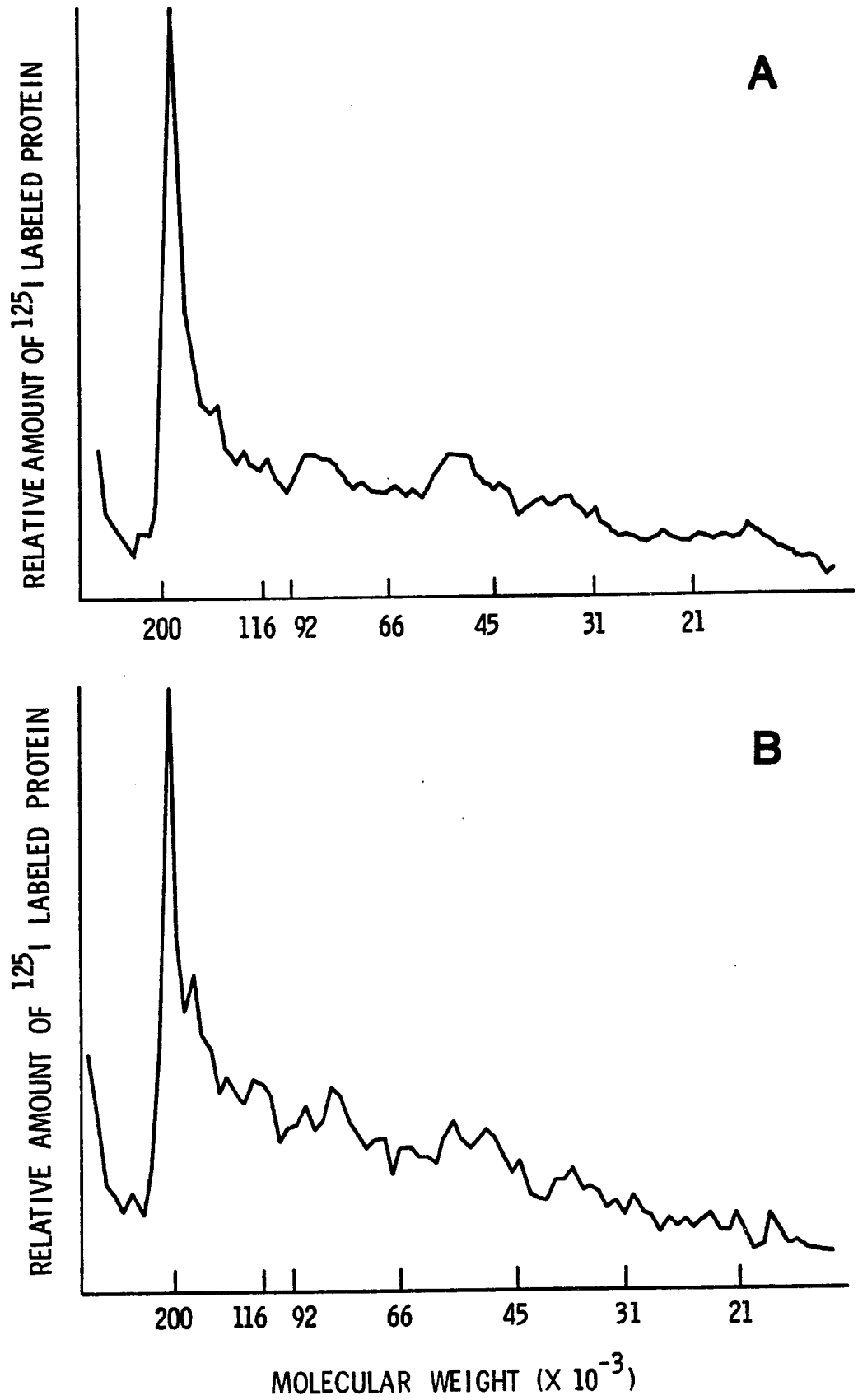
C
D
RELATIVE AMOUNT OF ^{125}I LABELED PROTEIN
200 116 92 66 45 31 21
MOLECULAR WEIGHT (X 10^{-3})

electrophoretic patterns were more similar to AKR-MCA than AKR-2B patterns. One such pattern obtained from TGF-treated cells is shown in Figure 2A.

. A typical electrophoretic pattern obtained 24 h after treatment of AKR-2B cells with EGF is shown in Figure 2D. The major peak of labeled proteins was observed at 200,000 daltons while a minor peak was found at mol. wt. 63,000. In sharp contrast, TGF-treated cells at 24 h showed an 82,000 molecular weight peak which was always larger than the 63,000 molecular weight peak; also, there was always a readily apparent peak with mol. wt. 53,000.

Figure 3 shows typical electrophoretic patterns of lactoperoxidase-^{125}I-labeled cell-surface proteins from both EGF- and TGF-treated cells 48 h post treatment. Although both EGF- and TGF-treated cells retained a transformed morphology at 48 h, significant changes occurred in the electrophoretic profile of TGF-treated cells compared to profiles at 24 h post treatment. The labeling pattern of TGF-treated cells was more similar to that of normal AKR-2B cells than to that of the permanently transformed AKR-MCA cells. The major peak of labeling at 48 h had a mol. wt. of 200,000. The peaks in the 50,000-90,000 mol. wt. region of those profiles associated with growth factor treatment at 24 h and with AKR-MCA cells were not discernable at 48 h. A shoulder of relatively significant labeling was found between the 200,000 and 116,000 mol. wt. markers. Most of this labeling was estimated to be at a mol. wt. greater than

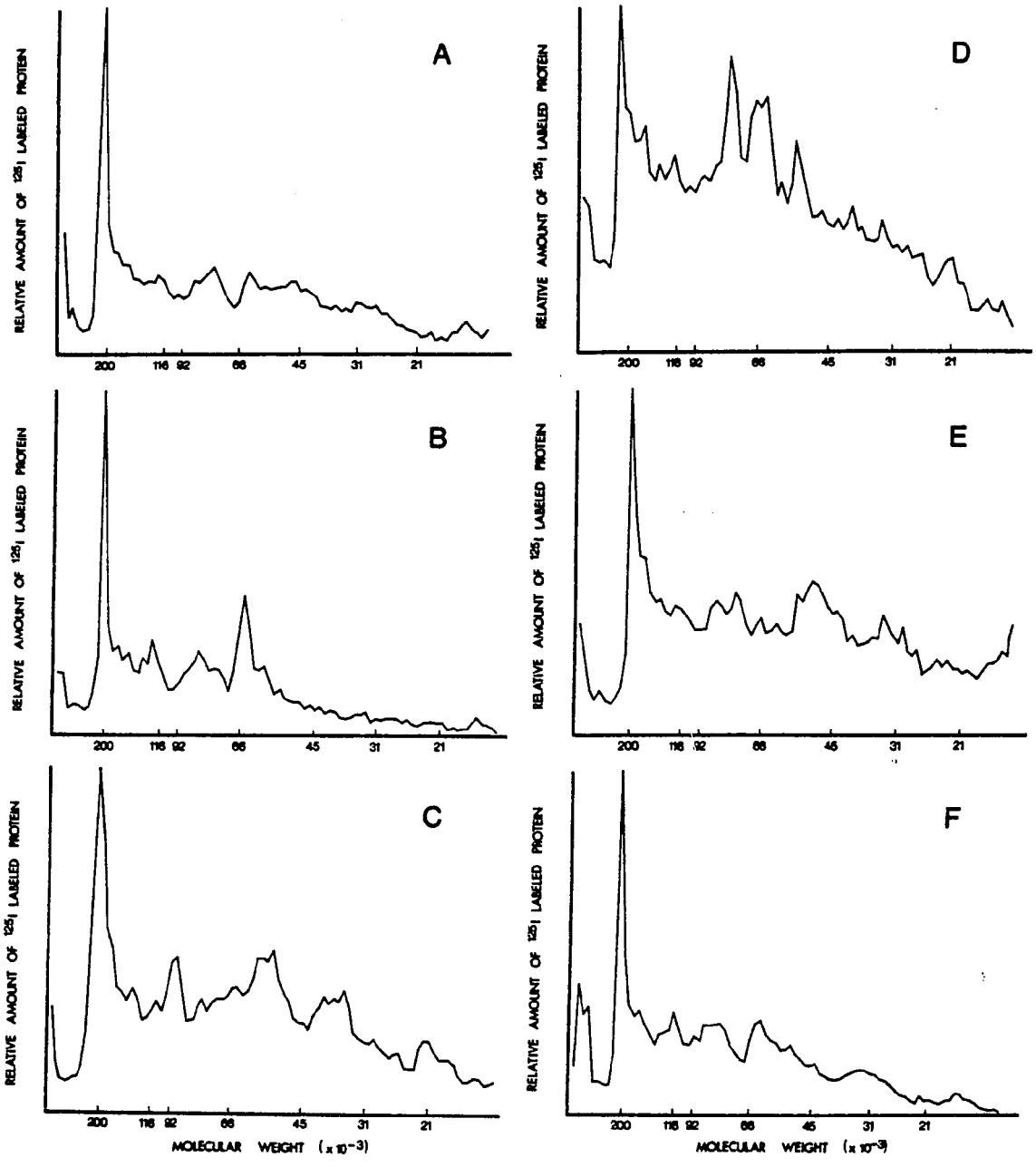
Figure 3 - Electrophoretic patterns of ^{125}I-labeled cell-surface proteins from AKR-2B cells radioiodinated 48 h after exposure to (A) 50 µg/ml crude TGF and (B) 50 ng/ml EGF.


A
RELATIVE AMOUNT OF ^{125}I LABELED PROTEIN
200
116
92
66
45
31
21
B
RELATIVE AMOUNT OF ^{125}I LABELED PROTEIN
200
116
92
66
45
31
21
MOLECULAR WEIGHT (X 10^{-3})

150,000. At 48 h post treatment the EGF cells also yielded electrophoretic patterns similar to those of AKR-2B cells.

The time course of altered membrane labeling of TGF-treated AKR-2B cells was studied in more detail (Fig. 4). Prior to 10 h post treatment there was little change in membrane labeling. At 13 h, labeling of material in the molecular weight range of ∿63,000 became more apparent. At 16-18 h after TGF treatment labeled surface proteins of approximately 55,000 and 82,000 mol. wt. became prominent. Relatively smaller amounts of labeling were observed between the 200,000 and 60,000 peaks. By 24 h post treatment large peaks of labeling were observed at mol. wt. 82,000 and 55,000. By 37 h post treatment resolution of labeled material at mol. wt. ∿63,000 was nearly lost and the peaks at mol. wt. 82,000 and 55,000 were greatly reduced in relation to the peak at mol. wt. ∿200,000, but were still readily discernable within the profile. Around 48 h a pattern similar to that of untreated AKR-2B cells was obtained which was similar to those described above for Figure 3. It is important to note in this experiment that at 24 h post treatment of AKR-2B cells there was still a significant amount of labeled material with mol. wt. 200,000 relative to the electrophoretic profiles of TGF-treated AKR-2B cells shown in Figure 2. Thus, in this experiment (Fig. 4) the electrophoretic profile of TGF-treated cells at 24 h is more similar to that of the AKR-MCA profile shown in Figure 2. The profiles from Figures 2 and 4 of cells at 24 h post treatment show the extremes

Figure 4 - Cell-surface response of AKR-2B cells to TGF as a function of time. Electrophoretic profiles of radioiodinated surface proteins from AKR-2B cells which were ^{125}I-labeled after being exposed to TGF-containing media for (A) 7 h; (B) 13 h; (C) 18 h; (D) 24 h; (E) 37 h; and (F) 45 h. The cells were treated with TGF, radiolabeled, solubilized, and electrophoresed as described in "Material and Methods."


A
B
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RELATIVE AMOUNT OF 125I LABELED PROTEIN
200
116
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21
MOLECULAR WEIGHT (x 10-3)

obtained in five radioiodinations from five different preparations of cells. Analysis of the total amount of label incorporated into membranes on a DNA basis indicates an average of 2.5 times more label was recovered in the 82,000 than in the 200,000 mol. wt. range of TGF-treated cells at 24 h post treatment (Table I).

Autoradiographs of radioiodinated cell-surface proteins from a second time course determination are shown in Figure 5. Electrophoresis in SDS was performed on an 5-10% polyacrylamide gradient gel for this experiment instead of the tube gel electrophoresis technique utilized for relative quantitation shown in Figure 4. The same amount of radiolabel was loaded onto each lane. The results are quite similar to those obtained from the tube gel electrophoresis system. Note in this experiment that relatively little material was labeled at mol. wt. 200,000 at 24 h post treatment with TGF.

Figure 6 represents a comparison of radioiodinated cell surface proteins from untreated AKR-2B and AKR-MCA cells by autoradiography after slab gel electrophoresis. The same amount of ^{125}I activity was applied to both lanes to provide for a direct comparison of the relative amounts of labeled proteins. As shown in Figure 6, the lane containing ^{125}I-labeled surface proteins from AKR-MCA cells did not reveal any material in the 200,000 mol. wt. region while the 55,000, 60,000 and 82,000 mol. wt. bands were prominent. The AKR-2B lane showed a very obvious band around the 200,000 mol. wt. marker and no 60,000 or 82,000 mol. wt. bands for the time

TABLE I - INCORPORATION OF ^{125}I INTO CELL-SURFACE COMPONENTS PER μg DNA AND FRACTION OF RECOVERED ^{125}I PRESENT IN THE 200 Kd AND 82 Kd PEAKS[1]

	(cpm/μg DNA) x 10^{-3}		% of recovered ^{125}I in:		
			200 Kd peak		82 Kd peak
AKR-2B	(4)[a]	2.77±0.76[b]	(5)[a]	13.2±2.7	ND[c]
AKR-2B treated for ∿24 h with TGF	(6)	16.54±4.44	(5)	6.4±1.5	16.4±5.1
AKR-MCA	(5)	1.51±0.40	(9)	11.1±4.4	19.5±4.2
AKR-2B treated for ∿24 h with EGF	(4)	4.11±3.00	(4)	15.1±2.5	5.1±1.0

1 ^{125}I activity reference dated.

[a] Number of experiments.

[b] Mean ± standard deviation.

[c] There was no clearly defined 82 Kd peak in the electrophoretic profiles of ^{125}I-labeled cell-surface proteins from AKR-2B cells, therefore, no determination of recovered ^{125}I in this region was possible.

Figure 5 - Autoradiograph of ^{125}I-labeled cell-surface proteins from AKR-2B cells which were treated with TGF for (A) 10 h; (B) 26.5 h; and (C) 52 h. The cells were labeled with ^{125}I after exposure to TGF for the indicated times, solubilized and electrophoresed on a 5-10% acrylamide gradient slab gel. Molecular weight standards migrated as indicated (mol. wt. X 10^{-3}). See "Material and Methods" for details.

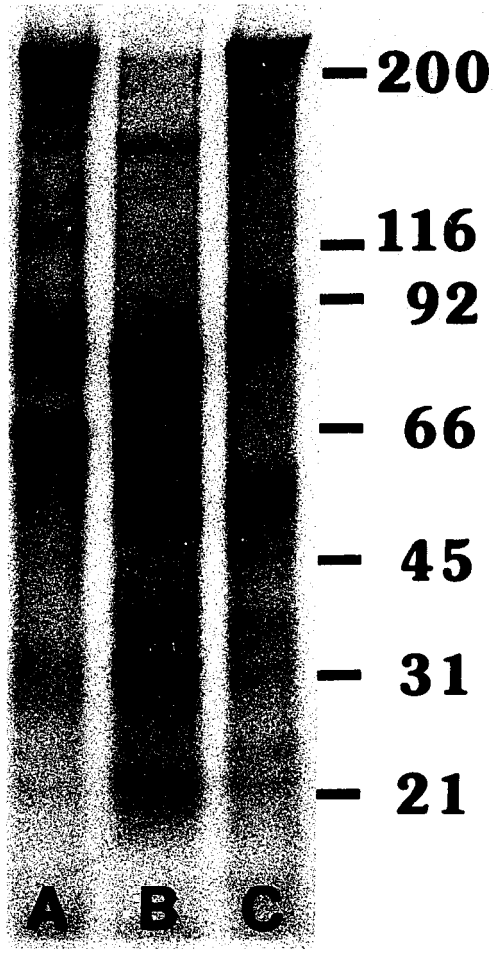
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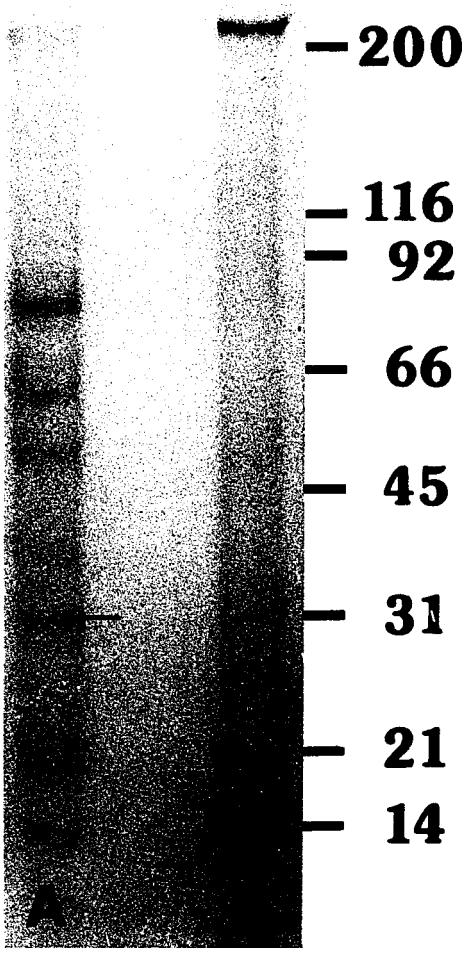
Figure 6 - Cell-surface proteins from (A) permanently, methylcholanthrene-transformed AKR-MCA cells and (B) non-transformed AKR-2B cells. Cells were labeled *in situ* and electrophoresed side-by-side on a 5-10% acrylamide gradient slab gel. Protein bands containing the ^{125}I label were visualized by autoradiography. Migration of molecular weight standards was as indicated (mol. wt. X 10^{-3}). Details are given in the text. An interesting feature observed upon autoradiography of slab gels on which material from AKR-MCA cells and TGF-treated AKR-2B cells was electrophoresed was a 30,000-31,000 mol. wt. ^{125}I-labeled cell-surface protein (indicated by the line) which has yet to be detected on AKR-2B cells not treated with TGF. While direct counting of sliced cylindrical gels on which labeled proteins have been electrophoresed allows for rapid and simple quantitative analysis, autoradiography is more sensitive and provides greater resolution. It is to this that we attribute the obscurity of the 30,000-31,000 mol. wt. protein peak in Figures 2 and 4 and its relative prominence in Figures 5B and 6A.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of exposure that was used. A characteristic of the AKR-MCA cell surface labeling pattern seen after autoradiography but not apparent in the tube gel electrophoretic profiles was the presence of a 30,000-31,000 mol. wt. species. This cell surface component was another distinguishing feature between AKR-MCA and AKR-2B cells.

An important question concerning the changes in membrane labeling described above is whether the differences in the profiles are due to reductions in the amount of labeled protein of mol. wt. 200,000 thus allowing for a greater proportion of the counts added to the gel to be proteins in the 50,000-90,000 molecular weight range. If so, this could lead to artifactually increased sensitivity for labeled proteins in the 50,000-90,000 molecular weight region of the electrophoretic patterns. Consequently, DNA determinations of labeled cells were made and total ^{125}I cell-surface membrane incorporation per μg DNA of labeled cell preparations was calculated. At 24 h following TGF exposure, levels of incorporation were ∿10 times higher than that of chemically transformed cells. EGF-treated cells had levels of incorporation similar to those of AKR-2B cells. Interestingly, the ^{125}I incorporation levels for AKR-MCA cells were approximately one-half of those of AKR-2B cells. These results (summarized in Table I) and the proportion of labeled protein of mol. wt. 200,000 found in treated cells indicate that there was a relatively small change in the amount of the 200,000 mol. wt. protein labeled per μg DNA, but that there were increases in

the amount of label incorporated into proteins responsible for the peaks in the 50,000-90,000 mol. wt. range in TGF-treated cells.

DISCUSSION

Significant differences between the SDS-PAGE profiles of radiolabeled cell surface proteins from malignant AKR-MCA cells and non-transformed AKR-2B cells were observed. AKR-2B cells had one major peak of radiolabeled material with mol. wt. 200,000. In addition to this peak, AKR-MCA cells showed a high level of labeling in the range of mol. wt. 50,000-90,000. We hypothesized that since the growth properties of AKR-MCA cells appeared to be related to their endogenously produced growth factors (Moses et al., 1978; Tucker et al., 1983), treatment of the non-transformed AKR-2B cells with TGF would induce similar alterations in the radioiodination of membrane proteins. The results indicated that treatment of AKR-2B cells with TGF did induce membrane alterations in AKR-2B cells which were similar to those seen in AKR-MCA cells. Treatment of AKR-2B cells with EGF provided a control for cell-surface alterations which might occur as a result of morphological changes or exposure to another mitogenic agent. Although EGF also induced membrane alterations in AKR-2B cells, these were different from those induced by TGFs.

The altered membrane electrophoretic patterns observed from TGF- or EGF-treated cells were transient. Alterations were observed at 24 h post treatment but the profiles had essentially returned to normal by 48 h post treatment. At least some of the TGF-induced alterations were probably not due to the morphological changes in AKR-2B cells. The transformed morphology of treated cells was maintained at 48 h post treatment despite changes in membrane labeling which indicated the restoration of an electrophoretic profile similar to that of untreated AKR-2B cells. The time-dependent rise and decline of labeling of AKR-2B cells in the 50,000-90,000 mol. wt. range indicates that exposure to TGF is not itself a sufficient stimulus to maintain the type of labeling pattern continuously exhibited by the malignant AKR-MCA cells. This suggests a basic difference in the abilities of the two cell types to respond to growth factors. Moses and his colleagues have previously suggested this possibility (Tucker et al., 1983).

Studies of the time course of the appearance of labeled material in the range of mol. wt. 50,000-90,000 indicated that material of mol. wt. 63,000 was the major peak prior to 20 h. At later times, the material with mol. wt. 82,000 dominated in the range of mol. wt. 50,000-90,000. By 36 h all labeling at mol. wt. 82,000, 63,000 and 53,000 was reduced in relation to the 24 h value, but there appeared to be a preferential reduction of labeling at mol. wt. 63,000. These results suggested that early membrane changes resulting from

TGF treatment were also the first alterations to disappear. Interestingly, the major alteration of membrane labeling induced by EGF was also at mol. wt. 63,000.

It should be reiterated that this work was performed with crude extracts of TGF obtained from conditioned medium. The material utilized was extracted with acetic acid and had a mol. wt. >3,500 as a result of dialysis. The activity was due to polypeptides in the crude extract since no alterations were observed with DTT or trypsin-treated extracts. However, it is entirely possible that the membrane alterations were the result of synergistic interactions between subpopulations of TGF or other polypeptide material in the crude extracts. The present study was performed with crude extracts because of the large amounts of material required for labeling studies and the small yield of TGF from conditioned medium.

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CELL SURFACE FEATURES ASSOCIATED WITH DIFFERENTIATION-INDUCTION OF METHYLCHOLANTHRENE-TRANSFORMED AKR-2B FIBROBLASTS BY N,N-DIMETHYLFORMAMIDE [1]

by

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ABSTRACT

Methylcholanthrene-transformed AKR-2B mouse embryonal fibroblasts (AKR-MCA cells) were examined for cell surface alterations after growth in culture medium containing N,N-dimethylformamide (DMF) using the lactoperoxidase-glucose oxidase radioiodination procedure with subsequent electrophoresis. DMF has been shown to induce maturational changes in a variety of transformed cells *in vitro* and has been reported to produce a more normal phenotype when applied to cultured AKR-MCA cells. The electrophoretic profile of ^{125}I-labeled surface proteins from AKR-MCA cells exhibited a prominent peak of labeled material with a molecular weight of approximately 85,000. After growth of AKR-MCA cells in medium containing DMF, the M_r 85,000 peak was substantially reduced, while there was a large increase in M_r 200,000 to 250,000 radioiodinated surface material. This cell surface labeling pattern was virtually identical to that of the non-transformed AKR-2B fibroblasts from which AKR-MCA cells were derived. The cell surface alterations observed upon exposure of AKR-MCA cells to DMF occurred as a function of time of growth in DMF and DMF concentration. Growth of AKR-MCA cells

in DMF resulted in a steady increase in cell surface ^{125}I incorporation up to the fourth day of exposure to DMF. At this time, the incorporation level was 22.9-fold greater than that for untreated AKR-MCA cells. Incorporation of radiolabel was decreased after the fifth and sixth days of AKR-MCA exposure to DMF. This trend was also manifested by AKR-2B fibroblasts grown in the presence of DMF. The data suggest that there was increased expression of the $\underline{M}_r$ 200,000 to 250,000 surface protein on both AKR-2B and AKR-MCA cells when grown in DMF. DMF also inhibited morphological transformation and the cell surface changes associated with transformation of AKR-2B cells by exogenous transforming growth factors.

ABBREVIATIONS

The abbreviations used are: DMF, N,N-dimethylformamide; AKR-MCA cells, AKR-2B mouse embryonal fibroblasts permanently transformed via methylcholanthrene treatment; TGF, transforming growth factors; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; PBS, phosphate-buffered saline [0.01 M sodium phosphate/0.154 M sodium chloride (pH 7.4)]; csp200, radioiodinated cell surface protein with a molecular weight of 200,000 to 250,000; csp85, ^{125}I-labeled cell surface protein with a molecular weight of 82,000 to 87,000; csp63, radioiodinated cell surface protein with a molecular weight of 63,000; PMSF, phenylmethylsulfonyl fluoride; FBS, fetal bovine serum.

INTRODUCTION

DMF is one of many compounds which have been investigated as agents capable of inducing a more differentiated phenotype when applied to cancer cells in vitro (4,5,7,8-10, 12,27). DMF has been shown to induce maturational changes in Friend leukemia cells (27), reduce tumorigenicity and clonigenicity of cultured human colon cancer cells (10), and induce terminal differentiation of human promyelocytic leukemia cells (5). These findings are consonant with the theory of neoplasia which suggests that some tumours occur as a function of abnormal differentiation (17,12). Most studies have used morphological or biological parameters to assess differentiation, and until recently, there has been scant regard to the molecular basis of maturation-induction or molecular changes associated with differentiation.

Cell surface proteins have been associated with the malignant biological cell properties of various cancer types (2,23,24,26). We have shown previously that distinct cell surface changes occur upon transformation of normal AKR-2B mouse embryonal fibroblasts with methylcholanthrene or TGF (22). AKR-MCA cells are considered to be malignant (22).

However, recent work from our group has shown that growth of AKR-MCA cells in DMF resulted in assumption of a phenotype similar to that of the untransformed parental AKR-2B cells (3). DMF induced malignant AKR-MCA cells to respond to normal growth controls as indicated by restoration of EGF mitogenic response, increased doubling time, loss of anchorage-independent growth, and reduction in saturation density (3). DMF also caused AKR-MCA cells to exhibit a culture morphology similar to that of AKR-2B fibroblasts. Moses et al. (25) have demonstrated that AKR-MCA cells elaborate TGF. Another recent study from our group has shown that growth of AKR-MCA cells in the presence of DMF resulted in increased levels of TGF activity in conditioned medium (19). The demonstrated biological responsiveness of AKR-MCA cells to DMF and the availability of the normal, nontransformed cells from which the AKR-MCA cell line was derived make AKR-MCA cells well suited as a model system for investigating molecular changes induced by DMF which might be correlated with differentiation to the normal AKR-2B phenotype. This study describes the cell surface alterations resulting from treatment of AKR-MCA cells with DMF.

MATERIALS AND METHODS

Materials. Lactoperoxidase, retinoic acid, glucose oxidase, mithramycin A, and DNA type I from calf thymus were purchased from Sigma Chemical Co. (St. Louis, MO). Carrier-free $Na^{125}I$ at a concentration of 100 mCi/ml was obtained from Amersham (Arlington Heights, IL). EGF was from Collaborative Research (Lexington, MA). Enzymobead radioiodination reagent and all reagents for SDS/PAGE, including molecular weight standards, were purchased from Bio-Rad (Richmond, CA). ACS grade DMF was obtained from Fisher (Pittsburgh, PA). Cell culture supplies were from both Grand Island Biological Co. (Grand Island, NY) and Flow Laboratories (McLean, VA). Cell cultures were maintained in 25-sq cm Corning disposable plastic flasks (Corning, NY).

TGF. Crude TGF (generously provided by Dr. A.E. Levine, Department of Pharmacology, Baylor College of Medicine) was isolated from Novikoff rat ascites fluid using methodology described elsewhere (18). TGF activity was confirmed using the soft agarose assay (19).

Cell Culture and Exposure of Cells to DMF, TGF, Retinoic Acid and EGF. AKR-2B and AKR-MCA cultures (obtained

from Dr. H.L. Moses, Department of Cell Biology, Mayo Graduate School of Medicine, Rochester, MN) were maintained at 37° and 5% CO_2 in McCoy's Tissue Culture Medium 5A supplemented with 10% heat-inactivated FBS (v/v) and gentamicin (52 μg/ml).

Cell cultures were grown to a stage at which confluency would be reached within 24 hr. At this point, control cultures of AKR-2B and AKR-MCA received a complete change of fresh medium containing 10% FBS. Other flasks of AKR-2B or AKR-MCA cells received 5 ml of fresh FBS-supplemented medium which contained EGF (50 ng/ml) or crude TGF (180 μg/ml) in the presence or absence of 1.0% DMF (v/v). Cells were incubated for 24 to 26 hr in the presence of the medium mixtures just described and then radioiodinated in situ. AKR-MCA cells were also grown in culture medium supplemented with 10% FBS and 1.0% DMF for 1 to 7 days before being ^{125}I labeled.

In a separate series of experiments, AKR-2B and AKR-MCA cells were exposed to 1.0% DMF 1 day after inoculation of culture flasks and then grown in the presence of DMF for 1 to 6 days prior to radioiodination in situ. The amount of ^{125}I incorporated per μg of DNA was then determined.

Cell Surface Radiolabeling. Radioiodination of cell surface proteins was accomplished essentially as described previously (22) with slight modification. Cell cultures were washed 3 times with PBS [0.01 M sodium phosphate/0.154 M sodium chloride (pH 7.4)] and incubated at room temperature

for 10 to 12 min in 3 ml of PBS containing 1.5 units of glucose oxidase, 10 units of lactoperoxidase, 30 mg of β-D-glucose, and 10 μl of $Na^{125}I$ solution. At the end of the incubation period, the reaction mixture was removed, and the cells were washed 3 times with PBS and then mechanically harvested in PBS. At this time, an aliquot of the cell suspension was examined microscopically for trypan blue exclusion. The remainder of the cell suspension was centrifuged, and the resulting cell pellet was solubilized in 2.5% SDS (w/v), 5% β-mercaptoethanol (v/v), and 3 mM PMSF by heating at 70° for 20 min with periodic vortexing. After the solubilization step, the samples were clarified by centrifugation, and the supernatants were stored at -70° until electrophoresis.

Collection of Shed Cell Surface Proteins. Cell cultures radioiodinated using the protocol described above were washed 3 times with serum-free McCoy's medium in situ and then incubated at 37° for 4 hr under serum-free McCoy's. Afterward, the medium was passed through a 0.2-μm filter, dialyzed against 0.01 M sodium phosphate/1 mM PMSF at 4° for 20 to 24 hr, and then exhaustively lyophilized. Lyophilized material was stored at -70° until electrophoresis, at which time it was solubilized in 2.5% SDS/5% β-mercaptoethanol/3 mM PMSF, heated at 70° for 20 min, and clarified by centrifugation, and the supernatant was applied to SDS/PAGE.

Radioiodination of FBS. Proteins contained in FBS were ^{125}I labeled with immobilized lactoperoxidase and glucose

oxidase (Enzymobead). After labeling, the reaction mixture was passed through a Sephadex G-10 column using PBS as eluant to separate unbound ^{125}I and Enzymobead from labeled protein. Material which eluted at the void volume was collected and stored at -70° until electrophoresis when SDS/β-mercaptoethanol was added to the sample to bring it to a final concentration of 2.5% SDS/5% β-mercaptoethanol. This solution was heated for 20 min at 70° and then applied to SDS/PAGE.

SDS/PAGE. Electrophoresis of radiolabeled cell surface material on 6% acrylamide cylindrical gels and on linear 5 to 10% gradient acrylamide slab gels with subsequent detection of the regions of the gels containing radiolabel was performed as described previously (22).

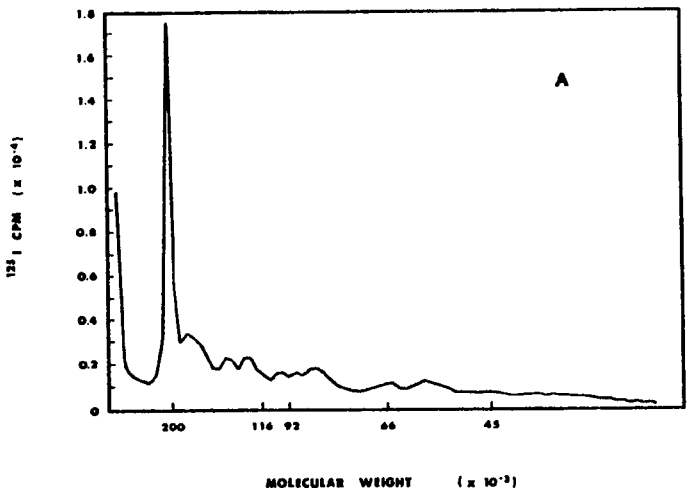
DNA Assay. Cells which were radioiodinated using the techniques described above were harvested in PBS, and the cellular DNA content was determined using the method of Hill and Whatley (14).

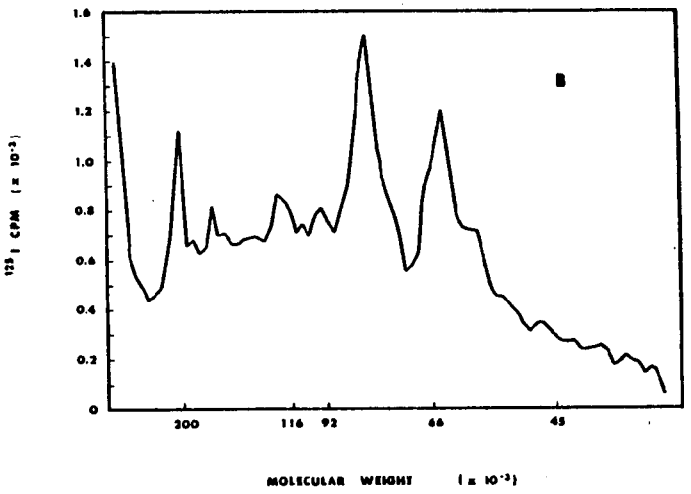
RESULTS

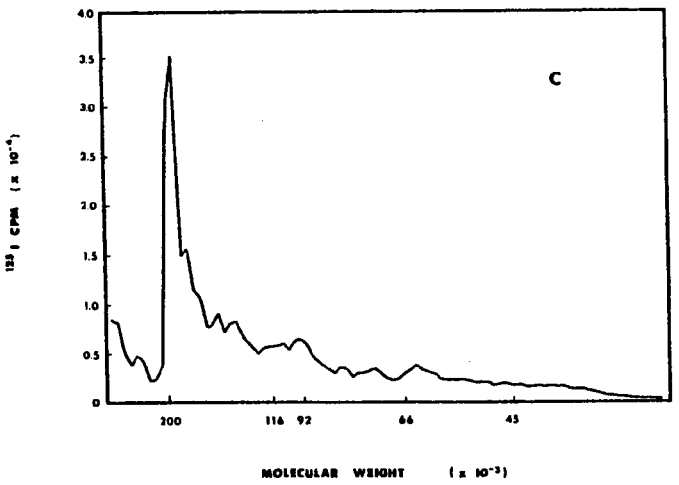
Electrophoretic profiles of radioiodinated cell surface proteins from AKR-2B and AKR-MCA are shown in Chart 1, A and B, respectively. As reported previously, the predominant radioiodinated AKR-2B cell surface component is csp200, while the single largest amount of ^{125}I-labeled surface material from AKR-MCA cells is csp85. In addition to the csp85 material, AKR-MCA cells also have a relatively large amount of csp63 susceptible to ^{125}I labeling and manifest a small amount of csp200. As shown in Chart 1D, the cell surface labeling pattern of AKR-MCA cells grown in the presence of 1.0% DMF is essentially the same as that of nontransformed AKR-2B cells. Exposure of AKR-MCA cells to DMF resulted in a dimunition of labeled csp85 and csp63 and an increase in ^{125}I-labeled csp200. Treatment of AKR-2B cells with DMF produced no discernible alteration in the electrophoretic profile of radioiodinated cell surface proteins (Chart 1C).

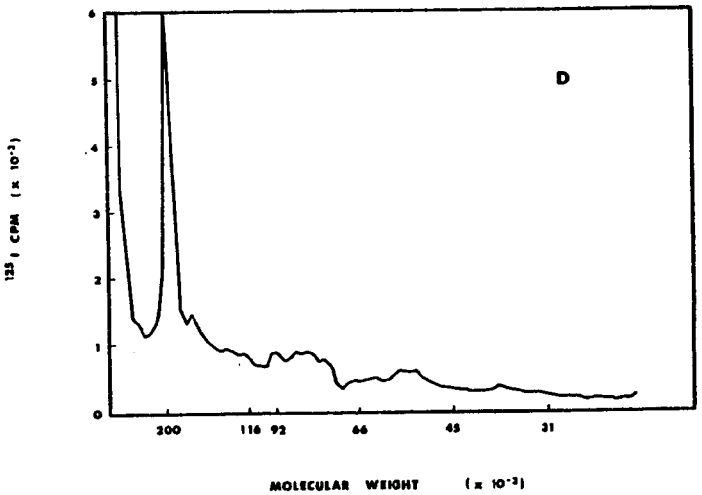
We elected to directly count sliced gels to determine the amount of ^{125}I present in gel sections in order to generate electrophoretic profiles indicating relative amounts of ^{125}I-labeled molecular weight species. While this is a

Chart 1 - Radioiodinated cell surface proteins separated by SDS/PAGE. The material was solubilized in SDS, β-mercaptoethanol, and PMSF and was from: AKR-2B fibroblasts (A); AKR-MCA cells (B); AKR-2B cells grown in 1.0% DMF for 7 days (C); and AKR-MCA cells grown in 1.0% DMF for 7 days (D). Electrophoresis was performed using 6% acrylamide cylindrical gels which were stained with Coomassie brilliant blue, destained, and sliced in 1-mm sections, and the gel slices were counted directly in a Beckman Model 4000 gamma counter.


A
^{125}I CPM (x 10^{-4})
MOLECULAR WEIGHT (x 10^{-3})
200
116
92
66
45


B
^{125}I CPM (x 10^{-3})
MOLECULAR WEIGHT (x 10^{-3})
200
116
92
66
45


C
^{125}I CPM (x 10^{-4})
MOLECULAR WEIGHT (x 10^{-3})
200
116
92
66
45


D
^{125}I CPM (x 10^{-3})
MOLECULAR WEIGHT (x 10^{-3})
200
116
92
66
45
31

rapid, quantitative procedure, slab gel electrophoresis with autoradiography provides greater visual resolution of bands. Consequently, an autoradiogram of ^{125}I-labeled cell surface proteins from AKR-MCA cells (Fig. 1A) and AKR-MCA cells grown in 1.0% DMF for 7 days (Fig. 1B) is presented for comparison to the profiles in Chart 1.

DMF may exert its cell surface effects via a solvent action. To control for this, we applied retinoic acid (a more physiological compound than DMF and one which is not a nonpolar solvent) to AKR-MCA cells at a concentration of 10^{-6} M for 7 days. Growth of AKR-MCA cells in retinoic acid produced a surface profile which closely paralleled those of AKR-2B fibroblasts and AKR-MCA cells grown in 1.0% DMF. Labeling profiles of AKR-MCA cells grown in retinoic acid are not presented because of their similarity to the surface labeling patterns of AKR-2B and DMF-treated AKR-MCA cells. AKR-MCA cells grown in 10^{-6} M retinoic acid became more flattened and cuboidal at culture confluence and grew in a monolayer fashion as did AKR-2B fibroblasts and DMF-treated AKR-MCA cells.

In an effort to determine whether or not the cell surface alterations of AKR-MCA cells associated with DMF treatment were dependent upon time of exposure to DMF, AKR-MCA cell surface proteins were radioiodinated after cell growth in the presence of DMF for various periods. The cell surface labeling patterns for AKR-MCA cells grown in 1.0% DMF for 0 to 5 days are shown in Chart 2. All culture flasks in this

Figure 1 - Autoradiogram of lactoperoxidase, ^{125}I-labeled cell surface proteins from AKR-MCA cells (Lane A) and AKR-MCA cells grown in the presence of 1.0% DMF for 7 days (Lane B). Molecular weight standards were run in adjacent lanes and migrated as indicated (molecular weight x 10^{-3}). The arrows point out the protein species which we have designated csp200 (upper arrow) and csp85 (lower arrow). An equal amount of radiolabel was applied to both lanes.

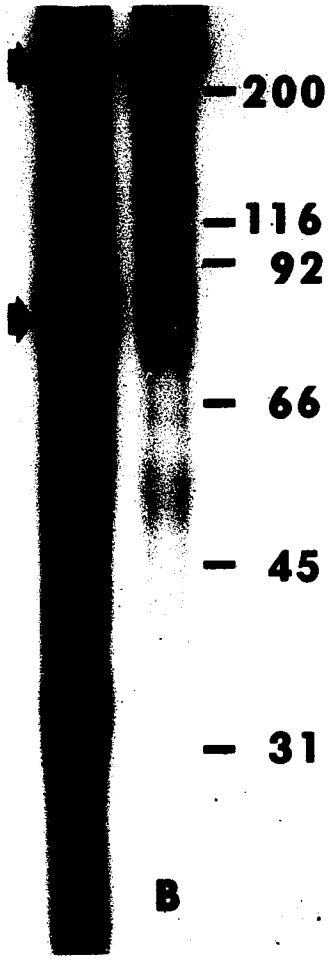
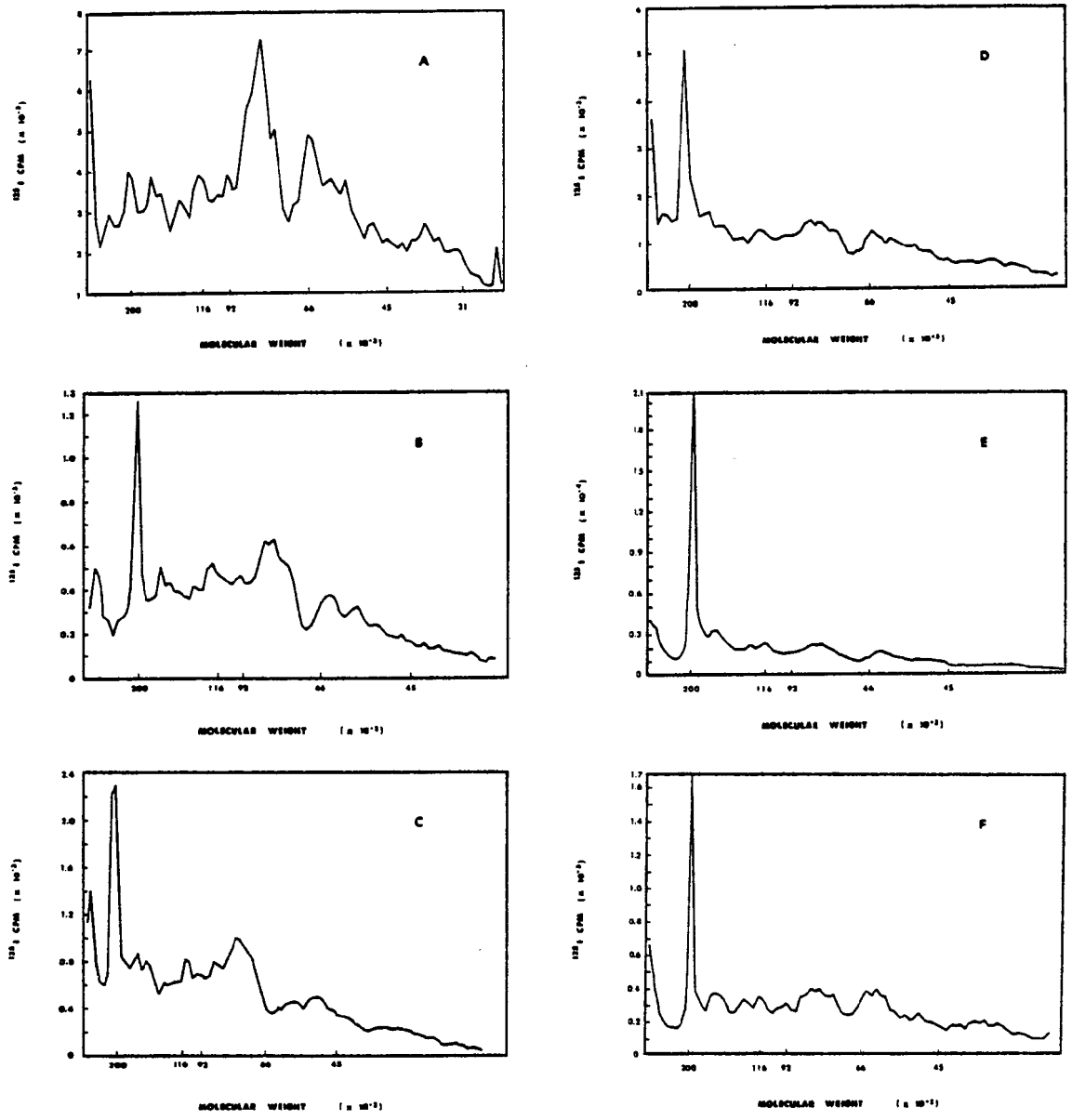

200
116
92
66
45
31
B

Chart 2 - Cell surface response of AKR-MCA cells to 1.0% DMF as a function of time. AKR-MCA cells were grown in culture medium containing 1.0% DMF for: 1 day (B); 2 days (C); 3 days (D); 4 days (E); and 5 days (F) prior to radioiodination. A is a representative electrophoretic pattern of labeled surface proteins from AKR-MCA cells which were not exposed to DMF.


A
B
C
D
E
F
MOLECULAR WEIGHT

experiment were initiated at the same time. After exposure to DMF for only 1 day, AKR-MCA cells showed a marked reduction in ^{125}I-labeled csp63 and csp85 and a concomitant increase in labeled csp200. This trend continued for Days 2 and 3 until Day 4, when the amount of ^{125}I-labeled protein in the $\underline{M}_r$ 45,000 to 116,000 region of the cell surface profile decreased to a level such that the electrophoretic pattern was virtually identical to the pattern of AKR-2B cell surface proteins (Chart 1A). Interestingly, after growth in DMF for 4 days, labeled $\underline{M}_r$ 45,000 to 116,000 protein began to increase, or alternatively, there was a relative decrease of radioiodinated csp200.

The amount of surface protein per cell available for radioiodination was reflected by the quantity of ^{125}I incorporated into proteins per μg of DNA. This was determined for both AKR-2B and AKR-MCA cells as a function of time of exposure to 1.0% DMF. The results are presented in Table I. The ^{125}I incorporation data for DMF-treated AKR-MCA cells are consistent with the cell surface events seen in the Chart 2 electrophoretic profiles. The greatest ^{125}I incorporation occurred after incubation of AKR-MCA cells in 1.0% DMF for 4 days (22.9-fold greater than untreated AKR-MCA cells). After 5 and 6 days in the presence of DMF, the incorporation level decreased. Similar changes in ^{125}I incorporation per μg of DNA were observed for DMF-treated AKR-2B cells. Even though untreated AKR-2B cells had more surface protein which could be ^{125}I labeled than did untreated AKR-MCA cells, the

Table I. Incorporation of ^{125}I into cell surface protein per μg DNA [1]

Time of growth in 1.0% DMF at time of labeling (days)	[(cpm / μg DNA) ± std. dev.] x 10^{-4}	
	AKR-2B	AKR-MCA
0	2.49 ± 0.13	0.69 ± 0.08
1	3.99 ± 0.57	0.61 ± 0.02
2	4.87 ± 0.43	4.42 ± 0.62
3		13.84 ± 0.08
4	11.94 ± 0.46	15.78 ± 0.34
5		10.53 ± 0.65
6	6.97 ± 0.91	6.12 ± 0.49

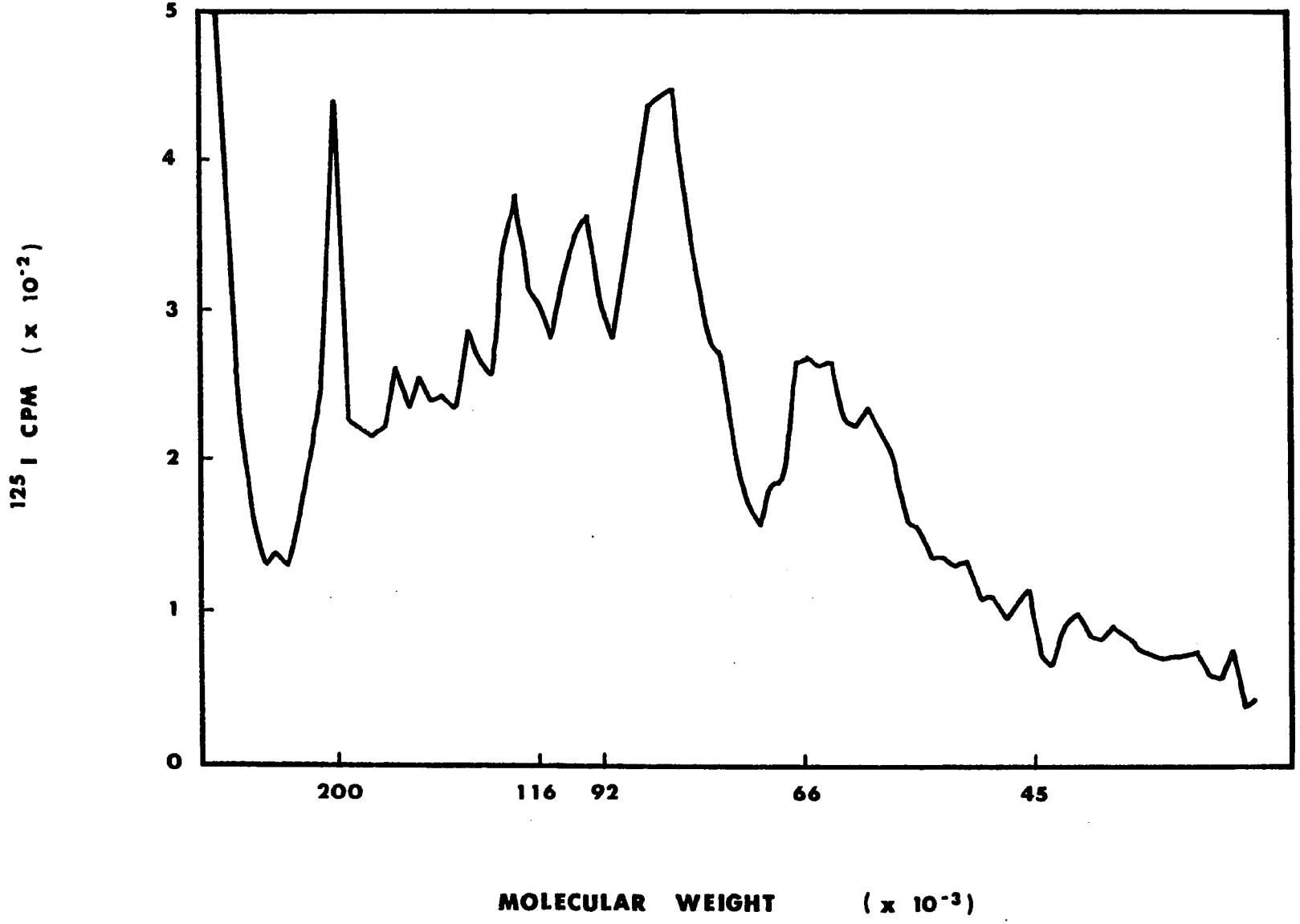
1 ^{125}I activity was reference dated to correct for radioactive decay.

2 cell lines had almost the same incorporation levels after growth in DMF for 2 days. Thereafter, the change in ^{125}I incorporation for AKR-2B cells paralleled the change for AKR-MCA cells.

To assess the influence of DMF concentration on the cell surface of AKR-MCA cells, AKR-MCA cultures were incubated in growth medium supplemented with 0.1% DMF for 24 hr. A representative electrophoretic profile of radioiodinated cell surface proteins from AKR-MCA cells exposed to 0.1% DMF is presented in Chart 3. It appears that, after growth in 0.1% DMF for 1 day, there was an increase in csp200 and in surface material with a molecular weight of 92,000 to 140,000, while csp85 was relatively decreased. However, the labeling pattern of AKR-MCA cells treated with 0.1% DMF is much more similar to the pattern of untreated AKR-MCA cells (Charts 1B and 2A) than to the pattern of AKR-MCA cells grown in 1.0% DMF for 1 day (Fig. 1B). The number of ^{125}I cpm per μg of DNA X 10^{-4} for AKR-MCA cells grown in 0.1% DMF-containing medium for 1 day was 0.79 ± 0.20 (S.D.).

To determine the effect of DMF on the ability of TGF to elicit surface changes in nontransformed cells, crude TGF was applied to AKR-2B cells, which had not been exposed previously to DMF, in fresh growth medium which also contained 1.0% DMF. Cells were treated with TGF in the presence or absence of DMF for 24 to 25 hr, the time required for TGF-induced surface alterations to become maximal (22), and then radioiodinated. The results of these experiments are shown

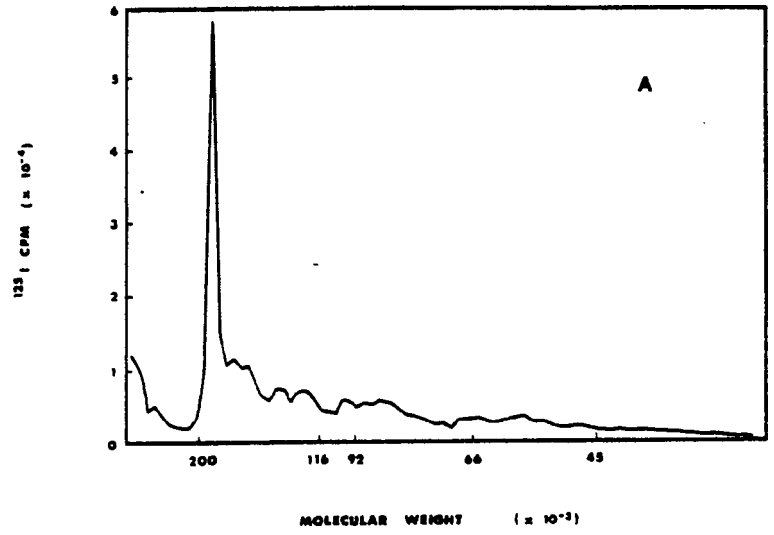
Chart 3 - Effect of 0.1% DMF on the cell surface labeling pattern of AKR-MCA cells. The cells were radioiodinated after growth in 0.1% DMF for 1 day.

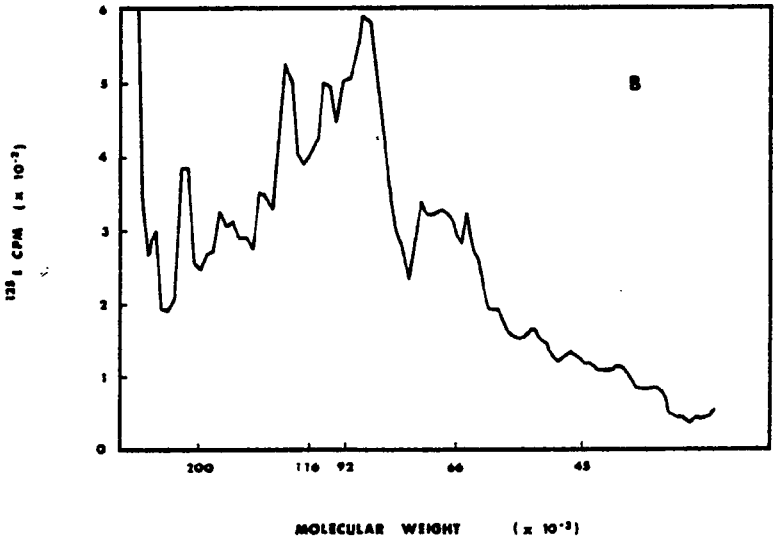

5
4
3
2
1
0
^{125}I CPM (x 10^{-2})
200
116
92
66
45
MOLECULAR WEIGHT (x 10^{-3})

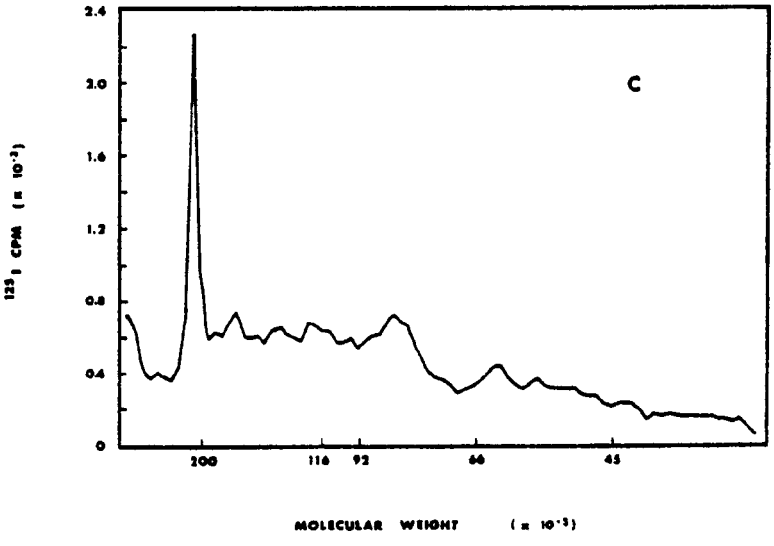
in Chart 4. Chart 4, A and B, represents controls for TGF treatment of AKR-2B cells. TGF-treated cells had as the most prominent electrophoretic peak ^{125}I-labeled $\underline{M}_r$ 80,000 to 86,000 surface material with significant peaks at molecular weights of approximately 250,000, 130,000, 90,000, and 68,000 (Chart 4B). This is in marked contrast to the cell surface protein profile of AKR-2B cells not treated with TGF, in which a single peak with a molecular weight of about 200,000 dominates the pattern (Chart 4A). Such is also the case for the labeling pattern of AKR-2B cells exposed to TGF and DMF simultaneously (Chart 4C), although here, there were discernible peaks of labeled material with molecular weights of ≈63,000 and 85,000. Along with the changes in the surface labeling profile, AKR-2B cells treated with TGF and 1.0% DMF at the same time did not manifest a transformed morphology.

The cell surface changes observed after growth of AKR-MCA cells in DMF and exposure of AKR-2B cells to TGF may have resulted from cell cycle variables. The following experiments were performed to evaluate the influence of cell cycle factors on the expression of csp200 and csp85. EGF was applied to AKR-2B cells as a control for the effect of mitogenesis on the cell surface labeling pattern. AKR-2B cells treated with EGF for 24 to 25 hr exhibited a relative increase in labeled csp63 as shown previously (22). Thus, an electrophoretic profile of ^{125}I-labeled cell surface proteins from EGF-treated cells is not presented here. Another control used was the addition of fresh medium containing 30%

Chart 4 - Cell surface manifestations produced by TGF treatment of AKR-2B cells (B) compared to the normal cell surface pattern of AKR-2B cells (A) and AKR-2B cells which were exposed to 1.0% DMF and TGF simultaneously (C).


A
^{125}I CPM (x 10^{-4})
0
1
2
3
4
5
6
200
116
92
66
45
MOLECULAR WEIGHT (x 10^{-3})

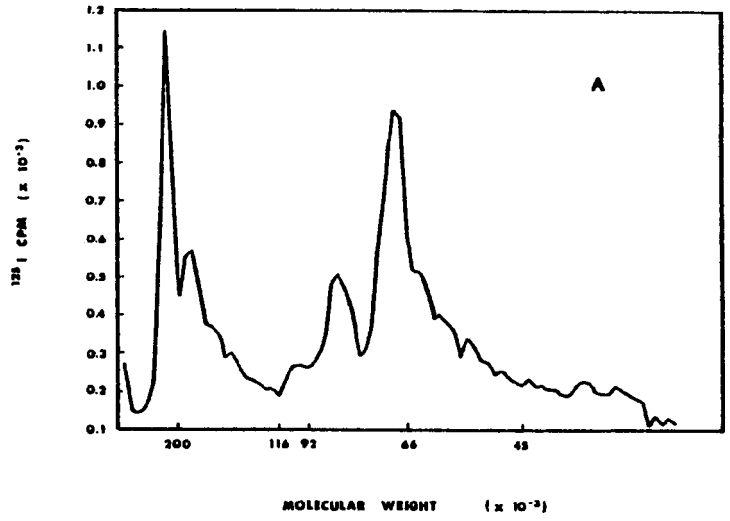

B
^{125}I CPM (x 10^{-2})
0
1
2
3
4
5
6
200
116
92
66
45
MOLECULAR WEIGHT (x 10^{-3})

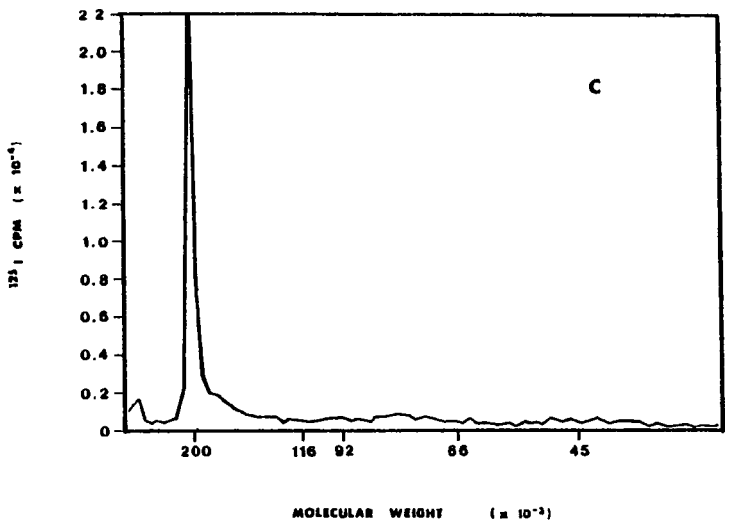

C
^{125}I CPM (x 10^{-2})
0
0.4
0.8
1.2
1.6
2.0
2.4
200
116
92
66
45
MOLECULAR WEIGHT (x 10^{-3})

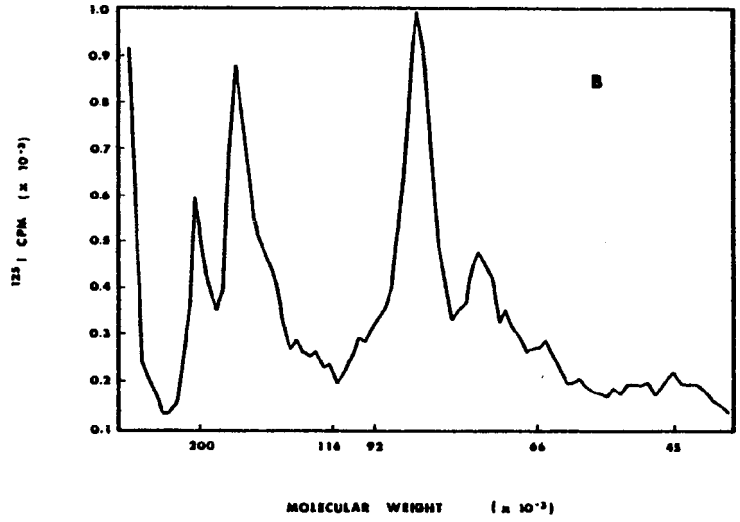
FBS. This excess of FBS failed to affect the surface labeling profile of AKR-2B fibroblasts. Furthermore, surface radioiodination of AKR-2B cells in log-phase growth yielded surface labeling profiles indistinguishable from the profiles of AKR-2B cells labeled at confluency. These findings suggest that the observed surface alterations are not cell cycle dependent.

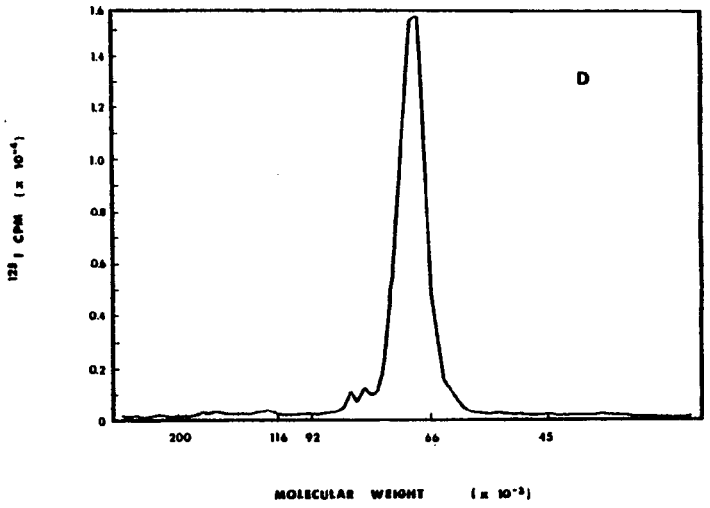
An important question regarding the cell surface effects of DMF on AKR-MCA cells is whether there is an actual decrease of csp85 material, or if this is merely artifactual and secondary to an increase in csp200 surface protein. Two considerations relevant to this matter are: the origin of the $\underline{M}_r$ 85,000 surface protein and what becomes of this material after it has been expressed on the cell's surface. The first of these points will be addressed in the "Discussion." As to the fate of csp85, it appears that it, as well as csp200, is shed from the surface of AKR-MCA cells (Chart 5B). The same is true for AKR-2B cells; however, while AKR-MCA cells shed csp85 as the single largest cell surface species, the single largest component shed from AKR-2B cells was csp200 (Chart 5A). Growth in 1.0% DMF produced a striking change in the composition of the proteins shed from the surface of AKR-MCA cells (Chart 5C). Virtually the only labeled surface protein shed from DMF-treated AKR-MCA cells was csp200. There was no detectable amount of csp85 shed from AKR-MCA cells exposed to DMF, indicating there was no csp85 on the cell surface, or if present, the csp85 could

Chart 5 - Cell surface proteins shed from: AKR-2B fibroblasts (A); AKR-MCA cells (B); and AKR-MCA cells grown in the presence of 1.0% DMF for 7 days (C). When applied to viable cells, the lactoperoxidase-glucose oxidase radioiodination technique is limited to externally oriented plasma membrane proteins. Therefore, only proteins shed from cell surfaces are depicted in the profiles. D presents the electrophoretic pattern of ^{125}I-labeled FBS as a control for serum proteins which may have been adsorbed to the cells, labeled, and later released into medium. It is important to note that the molecular weight markers are not in the same position for each panel.


A
200
116
92
66
45
MOLECULAR WEIGHT (x 10⁻³)


C
200
116
92
66
45
MOLECULAR WEIGHT (x 10⁻³)


B
200
116
92
66
45
MOLECULAR WEIGHT (x 10⁻³)


D
200
116
92
66
45
MOLECULAR WEIGHT (x 10⁻³)

not be shed. Radioiodinated FBS was electrophoresed as a control for proteins adsorbed from growth medium onto the cell surface and then shed into serum-free McCoy's medium. Chart 5D shows that essentially all ^{125}I-labeled FBS protein migrated between molecular weights equal to 66,000 to 70,000, suggesting that the shed radioiodinated material with molecular weights of 85,000 and 200,000 in Chart 5, A and B was produced by AKR-2B and AKR-MCA cells and that the large peak of $\underline{M}_r$ 68,000 protein in Chart 5A may have resulted from adsorbed growth medium which was labeled and later released. In addition to the $\underline{M}_r$ 85,000 surface protein, AKR-MCA cells shed a substantial amount of surface material with a molecular weight of 170,000 to 180,000. The electrophoretic profile of radioiodinated shed surface protein from AKR-2B cells also exhibits a $\underline{M}_r$ 170,000 to 180,000 peak but in a much reduced relative amount compared to AKR-MCA.

Cellular viabilities, based on trypan blue exclusion, of AKR-2B and AKR-MCA populations grown in the presence or absence of DMF were determined after radioiodination and mechanical harvesting and found to range between 88 and 98% with most being >94%.

DISCUSSION

Growth of permanently transformed AKR-MCA fibroblasts in culture medium containing DMF resulted in cell surface alterations which occurred in a similar but reverse manner to those surface changes which we have noted previously upon transformation of AKR-2B cells with TGF (22). The cell surface electrophoretic profile of DMF-treated AKR-MCA cells resembled the profile of AKR-2B cells. AKR-2B cells transformed via exposure to TGF manifested surface labeling patterns similar to those of AKR-MCA cells. TGF-treated AKR-2B cells have been shown to exhibit a time-dependent decrease of csp200 and increase of csp85, which is maximal 24 to 26 hr after TGF application (22). As presented in the current study, AKR-MCA cells expressed more csp200 and less csp85 when grown in the presence of DMF. These surface changes occurred as a function of time of exposure to DMF and DMF concentration. A more normal phenotype has been reported to result from application of DMF to AKR-MCA cells (3), while incubation of AKR-2B fibroblasts in the presence of TGF results in a transformed phenotype (22,25). Therefore, the

relative amounts of csp200 and csp85 on AKR-2B and AKR-MCA cells may be associated with the state of differentiation or transformation.

The ^{125}I incorporation data (Table I) and the electrophoretic profiles of the time series (Chart 2) indicate that the increase of the csp200 peak in the labeling patterns of DMF-treated AKR-MCA cells was a real increase in csp200 and not secondary to reduction in other surface components (e.g., csp85). Between the first and fifth days of exposure to DMF, there was a progressive increase in ^{125}I incorporation per AKR-MCA cell (as reflected by cpm/μg of DNA) and the fraction of recovered radiolabel present in the csp200 peak. In untreated AKR-MCA cells, 3.7% of the ^{125}I recovered after electrophoresis was accounted for by csp200. After growth of AKR-MCA cells in 1.0% DMF for 4 days, the time of greatest ^{125}I incorporation, the csp200 peak represented 20.5% of recovered label. Thus, at the time of maximal AKR-MCA cell surface response to DMF, there was a 5.5-fold increase in the fraction of recovered ^{125}I present in the M_r 200,000 to 250,000 region of the labeling patterns and a 22.9-fold increase in ^{125}I incorporation per cell.

It is possible that DMF treatment of AKR-MCA cells produced surface perturbations which preferentially exposed more of the csp200 molecule for radioiodination, and this was the cause of the increased csp200 peak and ^{125}I incorporation. Hager *et al.* (12) have commented on such a phenomenon as a possible mechanism for DMF-induced differentiation.

However, this concept is difficult to reconcile with the following observations: (a) similar response of AKR-MCA cells to retinoic acid and DMF; (b) smaller increase in ^{125}I incorporation for AKR-2B cells grown in DMF compared to AKR-MCA cells (4.8-fold versus 22.9-fold); and (c) decrease in csp200 levels (suggested by decreased ^{125}I incorporation) on both AKR-2B and AKR-MCA cells after exposure to DMF for 5 and 6 days.

These points are compatible with the idea that DMF and retinoic acid inhibit csp200 degradation and promote csp200 synthesis. Possible causes of the decrease in amount of csp200 on AKR-2B fibroblasts transformed by TGF or methylcholanthrene are: reduced synthesis; altered posttranscriptional modification so that csp200 or its precursor would not be inserted into the plasma membrane; and increased degradation. Increased degradation of csp200 is likely to be at least partially responsible for reduction in csp200 levels posttransformation, since TGF is able to induce a marked decrease in csp200 about 24 hr after application to AKR-2B cells (22). Hence, the observed increase in csp200 on AKR-MCA cells produced by DMF may be the result of both enhanced synthesis and inhibition of csp200 degradation.

The amount of csp85 distinguishes AKR-MCA cells pre- and post-DMF treatment and AKR-2B cells pre- and post-TGF application. Hynes (15) has suggested that membrane-associated proteins which appear upon transformation might be the products of the cleavage of larger proteins which are

decreased in the transformed state. This raises the possibility that csp85 may be derived from csp200 degradation in AKR-MCA cells and TGF-treated AKR-2B cells. A decrease of cell surface proteins with molecular weights of approximately 85,000 has also been connected with differentiation of human HL-60 promyelocytes. Working with dimethyl sulfoxide and retinoic acid, Felsted *et al.* (11) showed that a M_r 87,000 surface protein disappeared during differentiation of HL-60 cells. Chiao and Wang (4) demonstrated that a M_r 85,000 surface antigen became undetectable when HL-60 cells were induced to differentiate using conditioned medium from phytohemagglutinin-stimulated human lymphocytes.

The molecular weight of csp200 and the fact that AKR-2B and AKR-MCA are fibroblast lines suggest that csp200 is fibronectin. Preliminary results show that shed csp200 from both AKR-2B and AKR-MCA cells reacts with rabbit antiserum prepared against mouse fibronectin. Fibronectin, a cell surface glycoprotein, has been reported to become greatly reduced on a variety of cell types after transformation (see Refs. 15 and 30 for reviews). Loss of surface fibronectin has been linked to an increase of transformation-associated cell surface proteases (13,20,28). These observations are consistent with the idea that reduction of csp200 on transformed AKR-2B fibroblasts may be directly related to an increase in csp85. It would be interesting if csp85 were a fibronectin fragment as De Petro *et al.* (6) have described the ability of proteolytic fibronectin fragments to promote

morphological transformation *in vitro*. Fibronectin is capable of reversing certain cellular properties related to transformation (1,31) and inhibiting cell transformation (29) and has been proposed to be involved in differentiation (see Refs. 16 and 30 for reviews). Thus, DMF may induce differentiation in AKR-MCA cells through inhibition of the surface proteases responsible for degradation of csp200 and subsequent production of csp85.

Results from the current study also show that DMF inhibits the cell surface effects of TGF on AKR-2B fibroblasts, while a recent report by Levine *et al*. (19) described increased levels of TGF activity in medium in which DMF-treated AKR-MCA cells were grown. DMF may antagonize TGF metabolism in such a way as to influence TGF activity. Inhibition of TGF action may be the manner in which DMF affects csp200 and csp85 levels.

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N,N-DIMETHYLFORMAMIDE-INDUCED CELL SURFACE MODIFICATION AND ENHANCED SYNTHESIS OF A FIBRONECTIN-SIMILAR PROTEIN IN CULTURED HUMAN COLON CARCINOMA CELLS

MATERIALS AND METHODS

Materials. Lactoperoxidase, glucose oxidase, anti-rabbit IgG-FITC conjugate, mithramycin A, and DNA type I from calf thymus were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse fibronectin was purchased from Biomedical Technologies, Inc. (Cambridge, MA). Anti-human fibronectin and human fibronectin were from Collaborative Research (Lexington, MA). Carrier-free $Na^{125}I$ at a concentration of 100 mCi/ml was obtained from Amersham (Arlington Heights, IL). Rabbit immunoglobulins to human CEA were from DAKO Corp. (Santa Barbara, CA). All reagents and equipment for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE), including molecular weight standards, were purchased from Bio-Rad (Richmond, CA). ACS grade DMF was from Fisher Chemical Co. (Pittsburgh, PA). Cell culture supplies were obtained from Grand Island Biological Co. (Grand Island, NY) and Flow Laboratories (McLean, VA). Cell cultures were grown in 25-sq cm Corning disposable plastic flasks (Corning, NY). ^{125}I-labeled protein A with a specific activity of 26.0 μCi/μg was from ICN Biomedicals, Inc. (Irvine, CA).

Cell Culture. Stock cultures of the AKR-MCA cell line (provided by Dr. H.L. Moses, Department of Cell Biology, Mayo Graduate School of Medicine, Rochester, MN) were stored in liquid nitrogen. Working cultures of the line were maintained at 37° and 5% CO_2 in McCoy's Tissue Culture Medium 5A supplemented with 10% heat-inactivated fetal bovine serum (FBS) (v/v) and gentamicin (52 µg/ml). AKR-MCA cultures were discarded after being subcultured 10 times.

The HCT MOSER human colon cancer cell line was established *in vitro* from a spontaneously arising lesion as previously described (14). HCT MOSER cells were maintained in culture using the same conditions as those for AKR-MCA cells.

HCT MOSER cells were exposed to 0.1 - 0.5% DMF (v/v) in FBS-supplemented culture medium 1 day after inoculation into 25-sq cm culture flasks. Cells were grown in the presence of DMF for specified times prior to radioiodination *in situ*. During the times of DMF treatment, HCT MOSER cultures were not subcultured. Fresh DMF-containing medium was applied as necessary.

AKR-MCA and HCT MOSER cells were also grown in the presence and absence of DMF on glass microscope cover slips placed in 6-well culture dishes. Cells cultured in this manner were used for cell surface immunofluorescence studies. Consistent with past experimentation (61), AKR-MCA cells were grown in culture medium containing 1.0% DMF. AKR-MCA cells were exposed to DMF 4 days before immunofluorescent labeling while HCT MOSER cells were grown in DMF-containing medium for 21 days before labeling.

Preparation of Anti-Mouse Fibronectin. Rabbits were injected with an initial 150 µg of mouse fibronectin suspended in Freund's complete adjuvant. Approximately 75% of this quantity was injected subcutaneously in the neck region and the remaining 25% was injected intradermally in the flank. This injection procedure was repeated 1 week later with another 150 µg of mouse fibronectin suspended this time in Freund's incomplete adjuvant. One week after the second series of injections the rabbit received 100 µg of mouse fibronectin contained in phosphate-buffered saline (0.01 M sodium phosphate, 0.154 M sodium chloride, pH 7.4) (PBS). The injection sites and the proportions of material injected into each site were the same as for the first and second injection series. After the third round of injections, phlebotomy was performed using a heparinized syringe. The plasma was harvested and sodium azide was added to a final concentration of 0.1% (w/v). The plasma/sodium azide was divided into aliquots and stored at -20^{0}.

Cell Surface Radiolabeling. Radioiodination of cell surface proteins was accomplished using cultured cells *in situ* and methods previously detailed (61). Separate cultures of HCT MOSER cells subjected to 0.5% DMF were radioiodinated and harvested in PBS for DNA assay and subsequent determination of ^{125}I incorporation per µg DNA.

Collection of Shed Cell Surface Proteins and Excreted/Secreted Proteins. Radiolabeled cell cultures and cell cultures which were not labeled were washed 3 times with

FBS-free McCoy's medium. McCoy's medium was placed on the cells for 1 hr at 37°, removed and replaced with fresh McCoy's in which the cells were incubated for 23 hr at 37°. Serum-free McCoy's containing DMF was used in place of plain McCoy's for collection of shed cell surface proteins and excreted/secreted proteins from cells grown in the presence of DMF. After the 23 hr incubation the serum-free medium was collected, passed through a 0.2 μm filter, dialyzed against 5 μg/ml leupeptin at 4° overnight, exhaustively lyophilized and stored at -70°. Cell cultures were mechanically harvested in PBS for DNA assay after collection of the conditioned medium.

Lyophilized material was prepared for electrophoresis by solubilization in 2.5% SDS, 5% β-mercaptoethanol, 3 mM phenylmethylsulfonyl fluoride at 70° for 20 min with occasional vortexing followed by centrifugation. The supernatant was applied to SDS/PAGE.

As the lactoperoxidase-glucose oxidase radioiodination technique labels only the externally oriented plasma membrane proteins of viable cells (44), ^{125}I-labeled proteins contained in the serum-free conditioned medium must have been shed from cell surfaces. All other proteins were designated excreted/secreted proteins.

Immunofluorescent Labeling. Cells grown on glass cover slips were washed with PBS at 37° and then incubated in PBS, 1% bovine serum albumin (BSA) at 37° for 20 min. Three ml of fresh PBS, 1% BSA containing 50 μl of anti-human

fibronectin or 20 µl of anti-human CEA or 50 µl of anti-mouse fibronectin was placed on the cells for 20 min at 37°. The cells were then washed with PBS, 1% BSA at 22° followed by incubation for 20 min at 22° in 3 ml of PBS, 1% BSA in which there was 30 µl of anti-rabbit IgG-FITC conjugate. After exposure to the anti-IgG-FITC conjugate the cells were washed with PBS and viewed under a Nikon microscope equipped for fluorescence microscopy and photomicroscopy.

SDS/PAGE and Electrophoretic Transfer of Proteins to Nitrocellulose. Radioiodinated cell surface proteins and shed cell surface proteins were electrophoresed on 6% acrylamide cylindrical gels and on 5 to 10% linear gradient acrylamide slab gels using the method of Weber and Osborn (100). Cylindrical gels on which labeled material was electrophoresed were sliced at 1 mm intervals after being stained with Coomassie brilliant blue R-250 and destained, and counted in a Beckman Gamma 4000 gamma counter (Beckman Instruments, Inc., Irvine, CA). Those regions of slab gels which contained radiolabel were detected by autoradiography using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

Excreted/secreted proteins were electrophoresed on slab gels as above and then transferred onto nitrocellulose sheets (Bio-Rad, Richmond, CA) according to the procedure of Towbin et al. (94) using a Hoefer Scientific Instruments Transphor apparatus (San Francisco, CA). The nitrocellulose membranes onto which proteins were transferred were incubated in 5% BSA, 0.05% Tween 20 in PBS overnight followed by

incubation overnight in 50 ml of 1% BSA, 0.05% Tween 20 in PBS (BTP) containing 100 µl of anti-human fibronectin. After being washed with BTP, the nitrocellulose sheets were placed overnight in 50 ml of BTP to which ∿5 x 10^6 cpm of ^{125}I-labeled protein A was added. Following exposure to labeled protein A the sheets were washed with PBS, dried and autoradiographed. All washings and incubations were performed at 22°.

DNA Assay. Cellular DNA content was determined by the method of Hill and Whatley (43) using calf thymus DNA as standard.

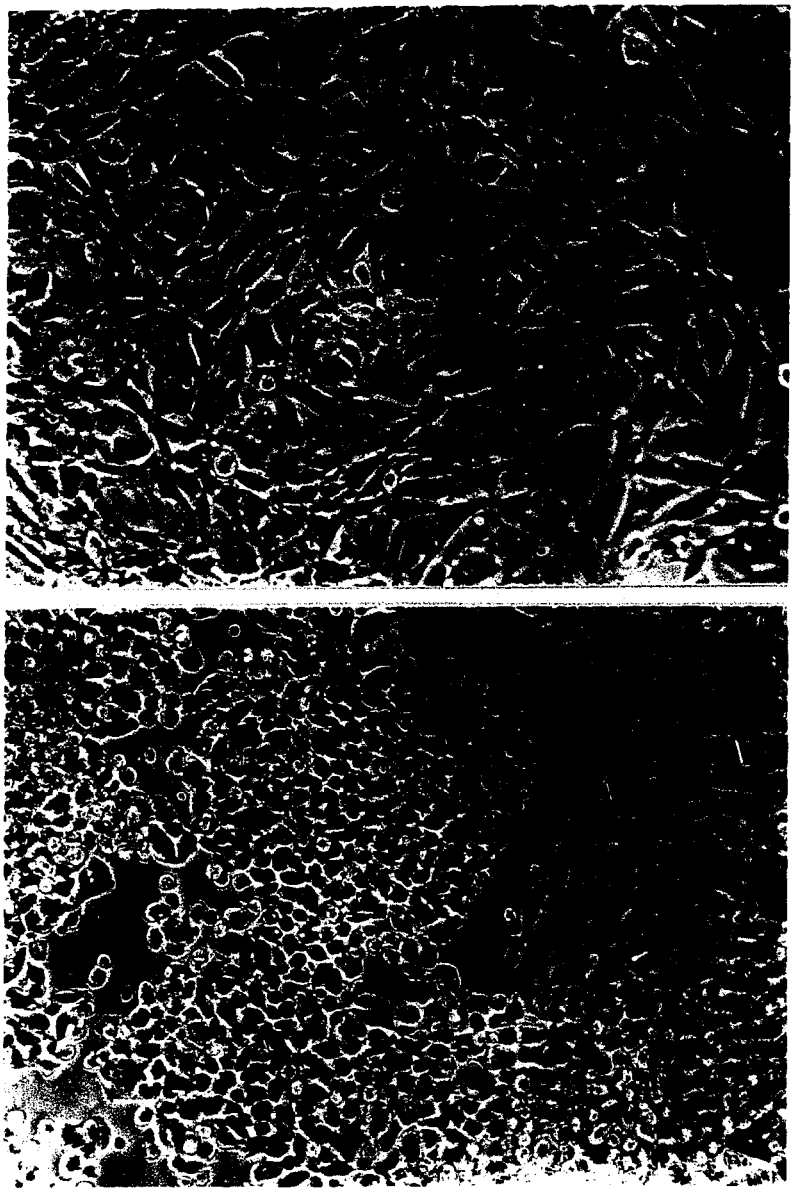
RESULTS

Growth of HCT MOSER cells in 0.5% DMF resulted in morphological changes which occurred in a time-dependent manner, as shown in Figure 1. HCT MOSER cells not exposed to DMF (Fig. 1A) exhibited a round shape *in vitro* and grew in cell clusters in which there were indistinct cell boundaries and in which many cells were not in contact with the flask. Continuous exposure of HCT MOSER cells to 0.5% DMF for 14 days yielded cells which were flattened and elongated (Fig. 1B). Exposure to DMF for an additional 7 days produced further flattening of HCT MOSER cells, complete attachment of cells to the flask, and acquisition of a fibroblastic appearance (Fig. 1C). The degree of confluency of HCT MOSER cultures treated with DMF was not a factor in DMF-induced morphological alterations. At culture confluence, HCT MOSER cells grown in DMF for 21 days manifested the same flattened, fibroblastic aspect (Fig. 2B) as subconfluent cultures (Fig. 1C). HCT MOSER cells in cultures approaching confluence (Fig. 2A) retained the same appearance as cells in markedly subconfluent cultures. Removal of DMF from HCT MOSER cells

Figure 1 - Effect of 0.5% DMF on HCT MOSER cell morphology. HCT MOSER cells were grown in 25-sq cm culture flasks in the absence of DMF (A) and in the presence of DMF for 14 days (B) and 21 days (C). X 100.

B
A

Figure 2 - Confluent cultures of HCT MOSER cells grown in the absence of DMF (A) and in the presence of 0.5% DMF for 21 days (B). X 100.



grown in DMF for 21 days resulted in reversion to a cellular morphology identical to that of HCT MOSER cells never exposed to DMF (Fig. 3). This morphological reversion occurred within 5 days after removal of DMF.

Cell surface changes associated with DMF treatment of HCT MOSER cells coincided with changes in morphology. Chart 1A is representative of the electrophoretic profiles of ^{125}I-labeled cell surface proteins from HCT MOSER cells. These profiles are characterized by distinct radiolabeled surface protein peaks of molecular weight 180,000 (csp180) and 120,000 (csp120). HCT MOSER cells also expressed a significant amount of surface material of approximately 87,000 molecular weight which was susceptible to labeling. Radioiodination of HCT MOSER cells grown in 0.5% DMF for 14 days revealed a decrease in csp120, relative increase in the two labeled protein species of 120,000 - 180,000 molecular weight, and a relative increase in csp180 (Chart 1B). The labeling patterns of HCT MOSER cells exposed to DMF for 21 days (Chart 1C) showed further reduction in csp120 and absence of 87,000 molecular weight protein. Electrophoretic profiles of HCT MOSER cells treated with DMF for 21 days were dominated by a single peak representing labeled surface protein of about 200,000 molecular weight (csp200) and resembled the labeling profiles of nontransformed IMR-90 human fibroblasts (Chart 1D).

Radioiodinated surface protein from IMR-90 fibroblasts, HCT MOSER colon carcinoma cells, and HCT MOSER cells grown

Figure 3 - Reversal of DMF-induced morphological alterations in HCT MOSER cells exposed to DMF for 21 days and then removed from the influence of DMF for 5 days. X 100.

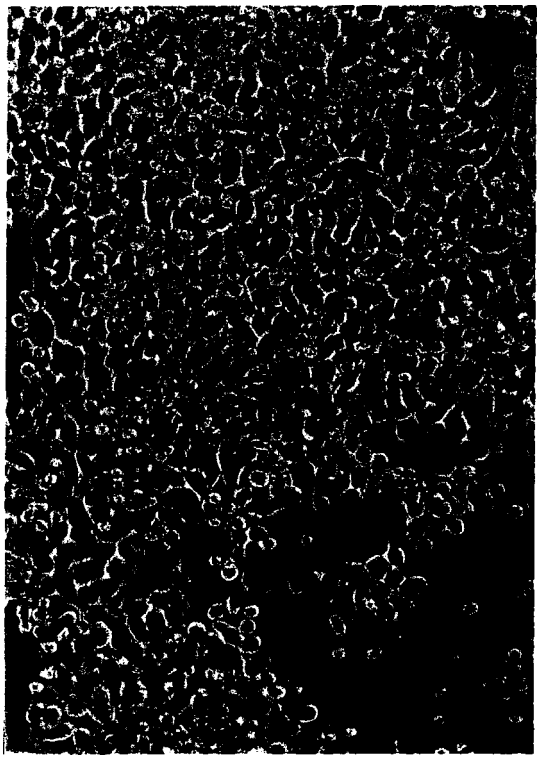
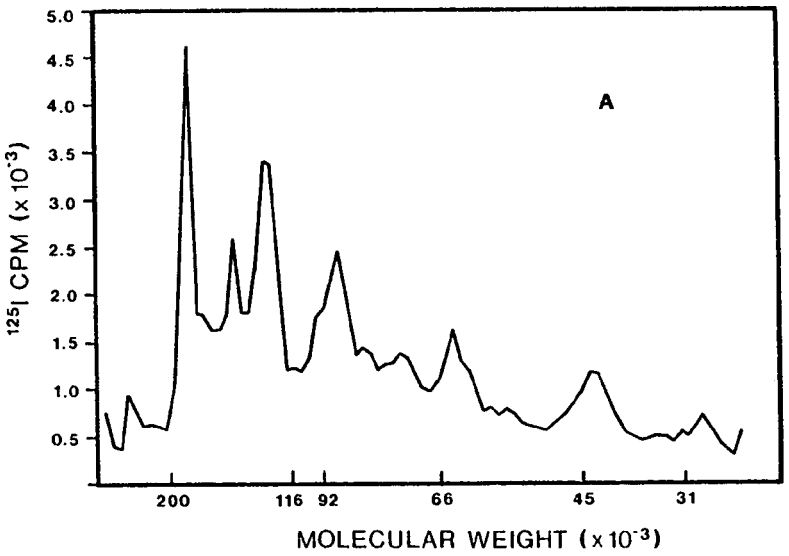
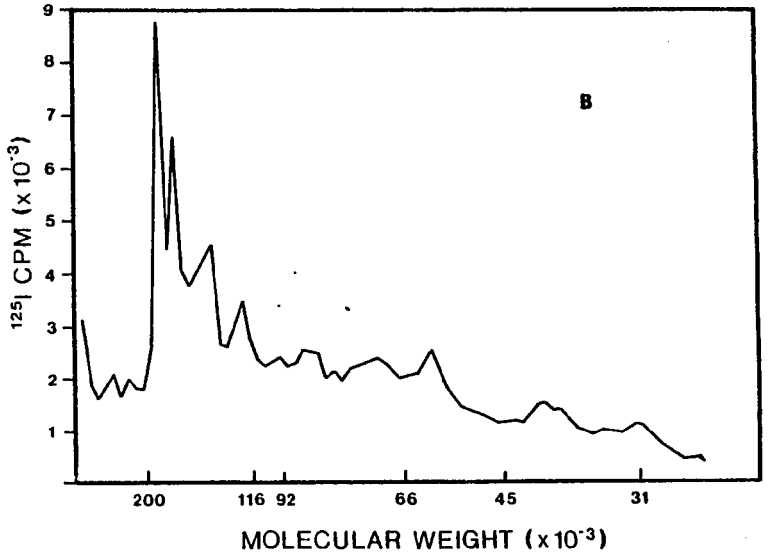
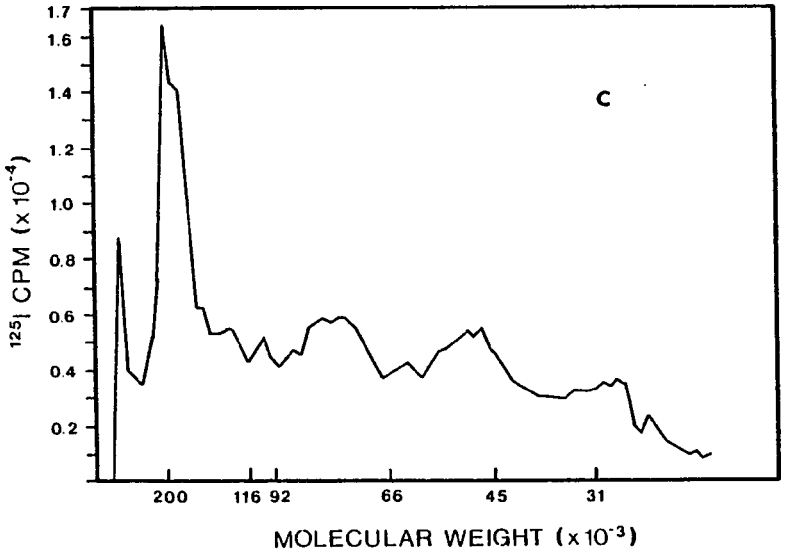
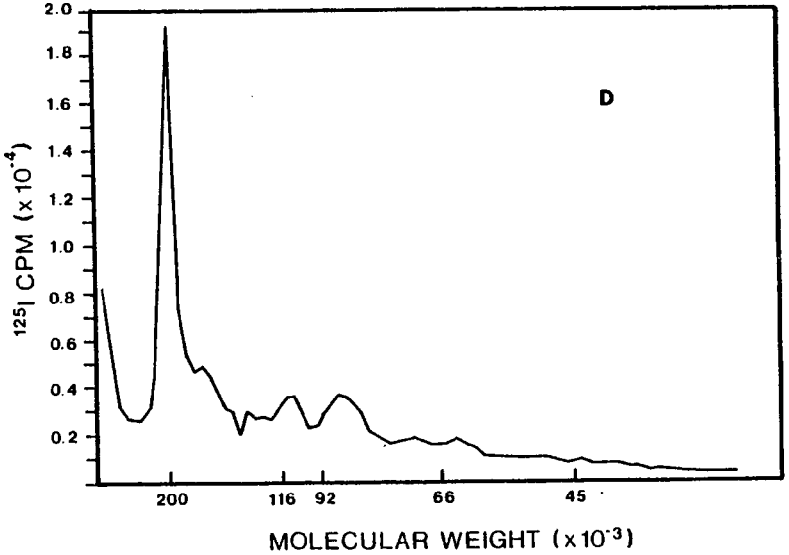


Chart 1 - SDS/PAGE fractionation of radioiodinated cell surface proteins from HCT MOSER cells labeled in situ and grown in 0.5% DMF-containing medium for 0 days (A), 14 days (B), and 21 days (C). The cell surface proteins of IMR-90 human fibroblasts were labeled, solubilized, and electrophoresed using the same methodology as for HCT MOSER cells. An electrophoretic profile of ^{125}I-labeled surface proteins from IMR-90 fibroblasts is shown in panel D.


A
^{125}I CPM (x 10^{-3})
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
200
116 92
66
45
31
MOLECULAR WEIGHT (x 10^{-3})


B
^{125}I CPM (x 10^{-3})
9
8
7
6
5
4
3
2
1
200
116 92
66
45
31
MOLECULAR WEIGHT (x 10^{-3})


C
^{125}I CPM (x 10^{-4})
1.7
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
200
116 92
66
45
31
MOLECULAR WEIGHT (x 10^{-3})


D
^{125}I CPM (x 10^{-4})
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
200
116 92
66
45
MOLECULAR WEIGHT (x 10^{-3})

for 21 days in 0.5% DMF was electrophoresed on slab gel followed by autoradiography for direct comparison of protein migration. The most prominently labeled cell surface component from IMR-90 (Fig. 4C) and DMF-treated HCT MOSER (Fig. 4B) cells appear to be of the same molecular weight. A radioiodinated protein of corresponding electrophoretic mobility is not apparent in the lane in which material from untreated HCT MOSER cells was run. (Fig. 4A).

In addition to the morphology reversion produced by removal of DMF from HCT MOSER cells, cells exposed to DMF for 21 days and then grown in the absence of DMF also exhibited a cell surface labeling pattern (Chart 2) very similar to that of untreated HCT MOSER cells (Chart 1A). Any differences between the Chart 1A and 2 profiles tend to be of a quantitative nature rather than qualitative.

Cell surface response of HCT MOSER cells to DMF was dependent on DMF concentration. HCT MOSER cells were grown for 21 days in medium containing 0.1%, 0.2%, and 0.3% DMF prior to radioiodination. Incubation in 0.1% DMF produced minimal cell surface changes (Chart 3A), but increasing the DMF concentration to 0.2% caused the appearance of a surface protein with a molecular weight greater than that of csp180. This surface protein was further increased when HCT MOSER cells were grown in 0.3% DMF (Chart 3C). Reduction of the csp120 and 87,000 molecular weight proteins, a prominent feature in the surface profiles of HCT MOSER cells subjected to 0.5% DMF, did not occur with the use of DMF concentrations

Figure 4 - Direct comparison of the electrophoretic mobilities of ^{125}I-labeled cell surface proteins from HCT MOSER cells (lane A), HCT MOSER cells grown in 0.5% DMF for 21 days (lane B), and normal IMR-90 fibroblasts (lane C). An equal amount of radiolabel was applied to each lane. Molecular weight standards migrated as indicated (molecular weight x 10^{-3}).

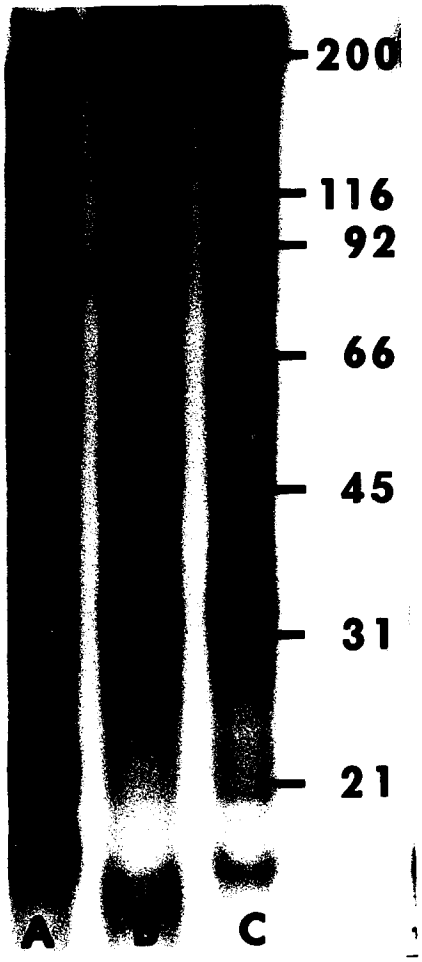

200
116
92
66
45
31
21
A
B
C

Chart 2 - Reversal of the cell surface effects of DMF. Cell surface electrophoretic pattern of HCT MOSER cells grown in 0.5% DMF for 21 days and then grown in the absence of DMF for 5 days.

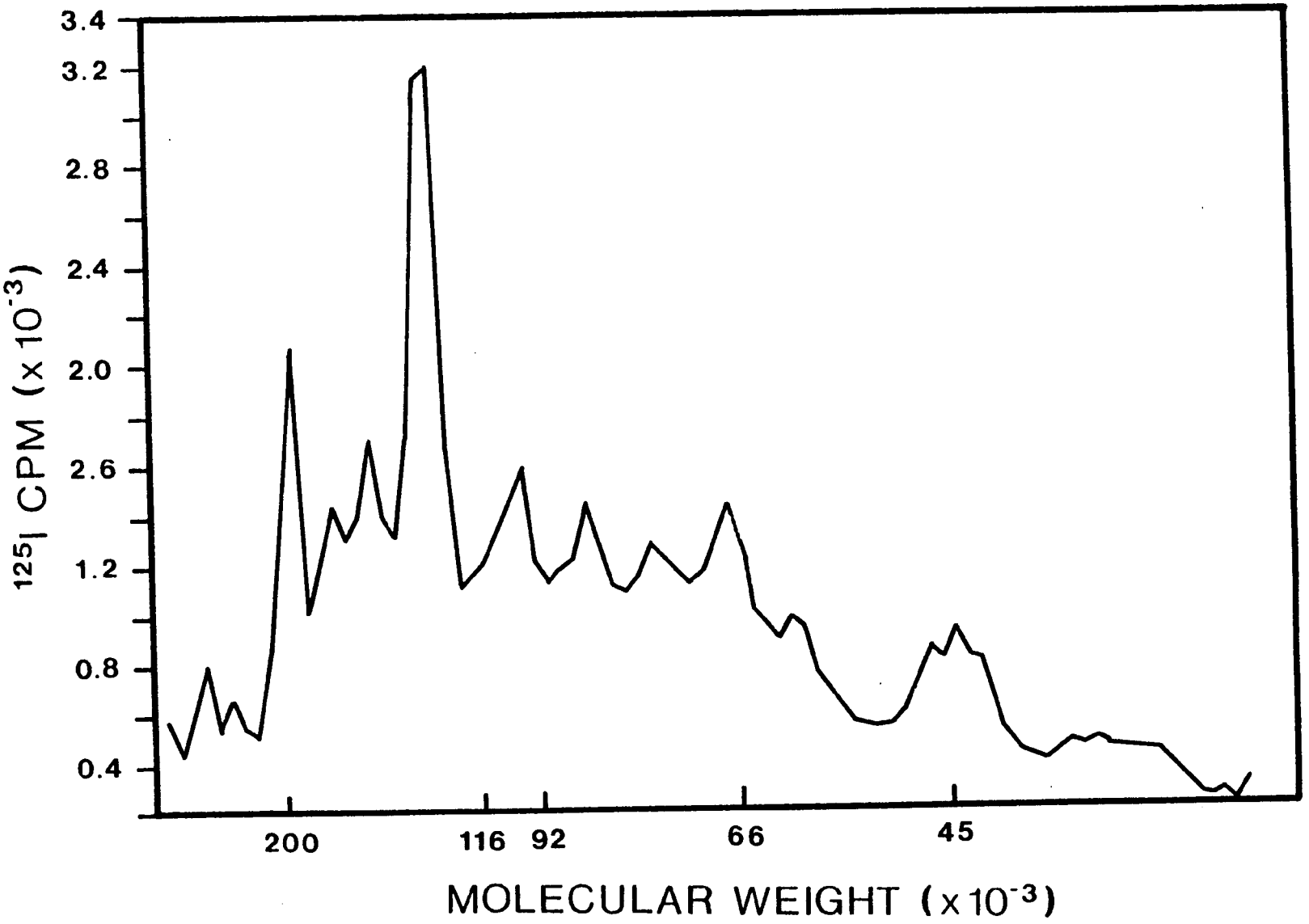
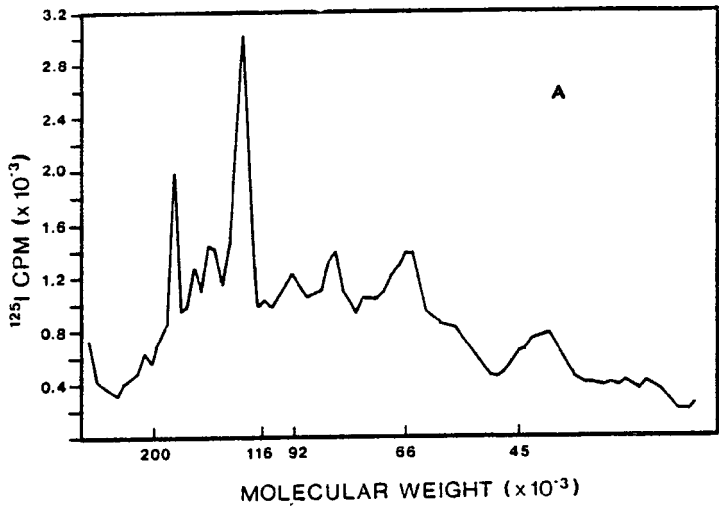
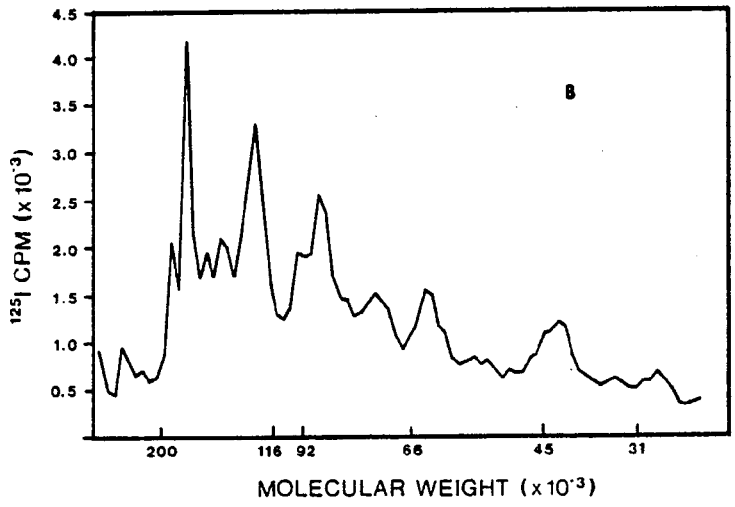
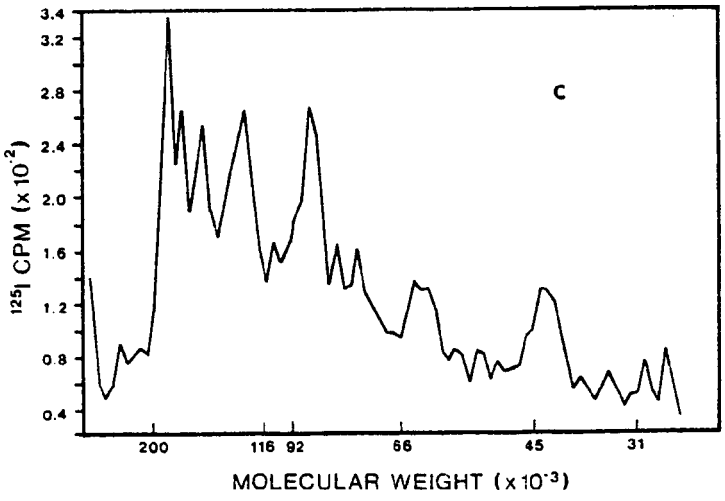

3.4
3.2
2.8
2.4
2.0
2.6
1.2
0.8
0.4
^{125}I CPM (x 10^{-3})
200
116
92
66
45
MOLECULAR WEIGHT (x 10^{-3})

Chart 3 - DMF concentration dependence of HCT MOSER cell surface alterations. HCT MOSER cells were exposed for 21 days to 0.1% DMF (A), 0.2% DMF (B), and 0.3% DMF (C) and then radiolabeled with ^{125}I.


A
^{125}I CPM (x 10^{-3})
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
200
116
92
66
45
MOLECULAR WEIGHT (x 10^{-3})


B
^{125}I CPM (x 10^{-3})
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
200
116
92
66
45
31
MOLECULAR WEIGHT (x 10^{-3})

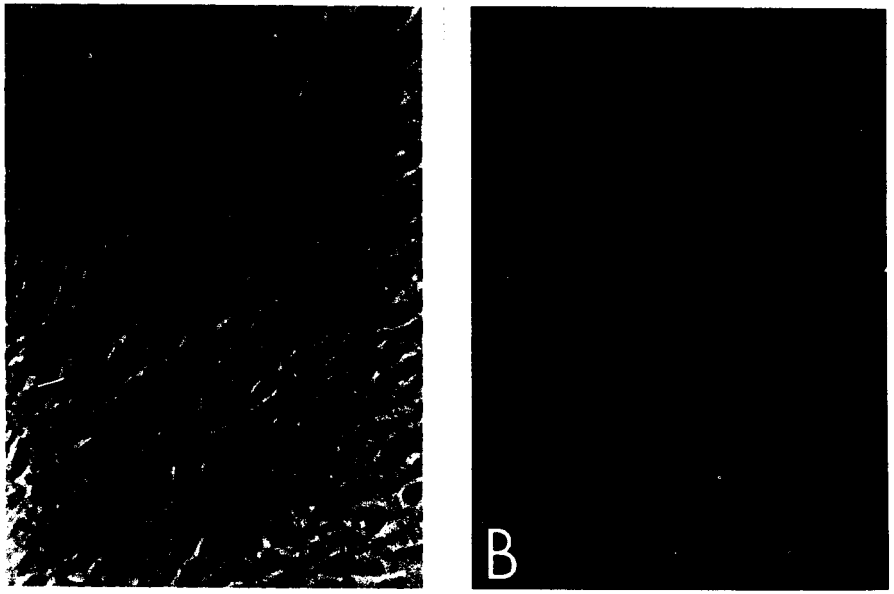

C
^{125}I CPM (x 10^{-2})
3.4
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
200
116
92
66
45
31
MOLECULAR WEIGHT (x 10^{-3})

$\leq$ 0.3%. However, there was a tendency for csp180 to decrease when HCT MOSER cells were exposed to 0.3% DMF.

The possibility that growth of HCT MOSER cells in DMF induced the elaboration of fibronectin was suggested by the appearance of a DMF-related surface protein susceptible to radioiodination which comigrated with the single largest labeled surface protein from IMR-90 fibroblasts and the effect of DMF on HCT MOSER cell morphology. Similar morphological and cell surface events were observed when transformed AKR-MCA fibroblasts were grown in DMF (61). The identity of a 200,000 - 250,000 molecular weight surface protein which was increased on AKR-MCA cells post-DMF treatment and decreased on AKR-2B cells upon transformation has been postulated to be fibronectin (59,61). Cell surface immunofluorescence techniques were employed to explore the possibility that csp200 from DMF-treated HCT MOSER cells and the 200,000 - 250,000 molecular weight surface protein of AKR-MCA cells exposed to DMF might be fibronectin.

AKR-MCA and HCT MOSER cells were incubated in anti-mouse and anti-human fibronectin, respectively, and then exposed to the appropriate anti-IgG-FITC conjugate. DMF-treated AKR-MCA and HCT MOSER cells were also exposed to anti-fibronectin and anti-IgG-FITC. Figure 5 shows that before growth in DMF, AKR-MCA cells expressed little surface material reactive with anti-mouse fibronectin. Growth in DMF-containing medium for 4 days resulted in induction of material on the surfaces of AKR-MCA fibroblasts which was

Figure 5 - Phase-contrast photomicrograph (A) of AKR-MCA cells incubated in antisera to mouse fibronectin prepared in rabbit followed by exposure to anti-rabbit IgG FITC conjugate and fluorescence (B) of the same cells.


B

strongly reactive with anti-mouse fibronectin and which had a fibrillar pattern (Fig. 6B). Similarly, prior to DMF-exposure HCT MOSER cells evidenced no detectable surface fibronectin (Fig. 7), but growth in DMF-supplemented medium also resulted in the presence of a surface component immunoreactive with anti-fibronectin (Fig. 8B).

Contaminant fibroblasts in HCT MOSER cultures may have been selected for and allowed to proliferate secondary to exposure of the colon cancer cell cultures to DMF. This would account for the presence of cells with surface constituents reactive with anti-fibronectin. However, cells comprising DMF-treated HCT MOSER cultures continued to produce surface CEA as indicated by surface fluorescence after incubation in anti-human CEA and anti-IgG-FITC (Fig. 9B). This suggests that the cells comprising DMF-treated HCT MOSER cultures were of colonic origin.

HCT MOSER cells and HCT MOSER cells subjected to DMF were assayed for the amount of surface protein per cell available for radioiodination. A measure of this was obtained by determining ^{125}I incorporation per μg DNA. The results of this determination are presented in Table 1. As indicated, there was relatively little change in the amount of ^{125}I-labeled surface protein per cell before and after exposure to DMF.

It has been shown that cell surface protein of transformed cells is shed or released (12,18,61). Bystryn and Perlstein (18) have proposed that shedding of surface

Figure 6 - Phase-contrast image (A) of AKR-MCA cells grown in 1.0% DMF for 4 days and the fluorescence pattern (B) of surface fibronectin on the same cells. Immunofluorescence was performed as described in "Materials and Methods."

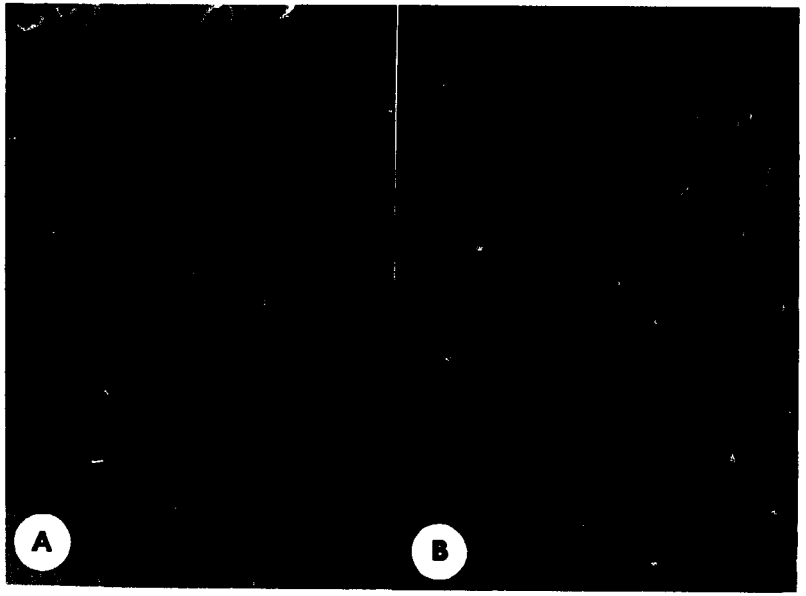

A
B

Figure 7 - Phase-contrast (A) and fluorescence (B) images of HCT MOSER cells incubated in anti-human fibronectin followed by exposure to anti-rabbit IgG FITC conjugate.

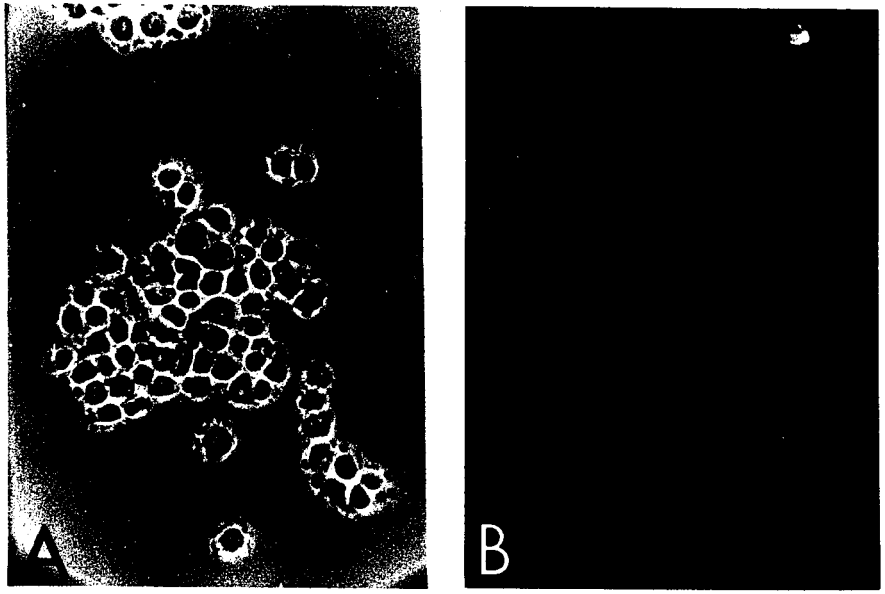

A
B

Figure 8 - Expression of surface fibronectin on HCT MOSER cells treated with DMF for 21 days. Phase-contrast (A) and fluorescence (B) photomicrographs of cells labeled with anti-human fibronectin and anti-IgG-FITC.


A
B

Figure 9 - Demonstration of the presence of surface CEA on HCT MOSER cells exposed to DMF for 21 days. Phase-contrast (A) and fluorescence (B) images of cells labeled with anti-CEA followed by anti-IgG-FITC.

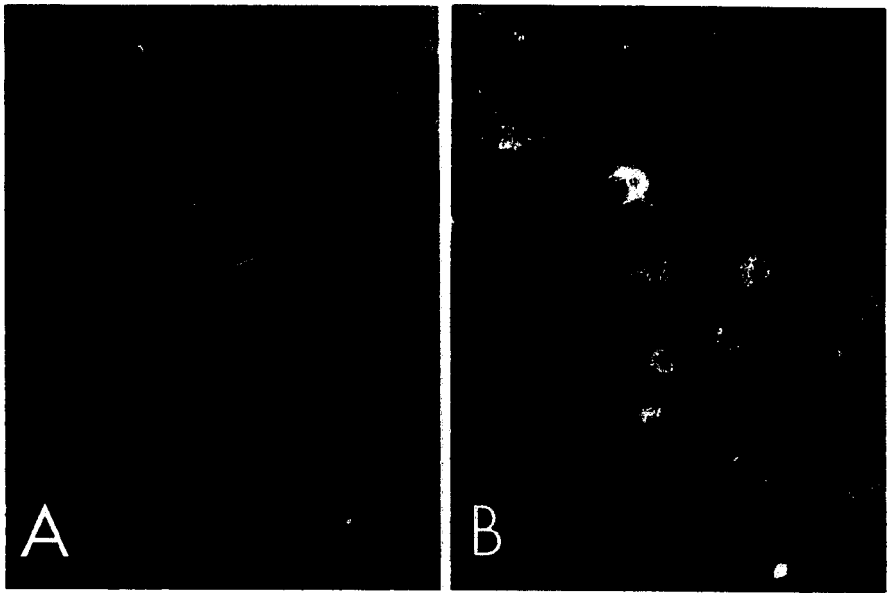

A
B

Table I - Incorporation of ^{125}I into HCT MOSER cell surface protein per µg DNA [1]

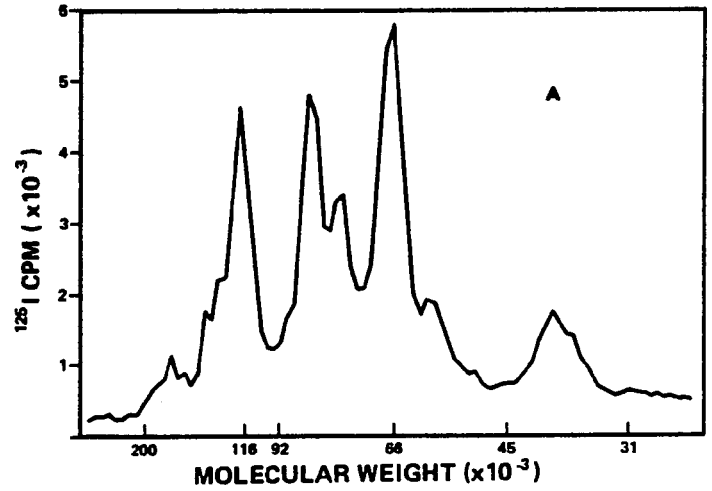
Time of growth in 0.5% DMF at time of labeling (days)	[(cpm/µg DNA) ± std. dev.] x 10^{-5}
0	1.35 ± 0.05
14	1.27 ± 0.20
21	1.45 ± 0.15
28	1.48 ± 0.16

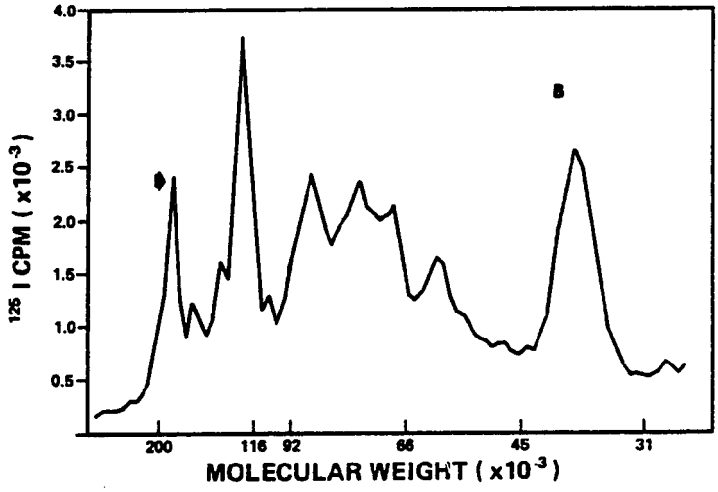
[1] ^{125}I activity was reference dated to correct for radioactive decay.

macromolecules from tumor cells is one pathway for the turnover of surface protein. In consideration of this, HCT MOSER cells were examined for differences in shed cell surface protein composition resulting from exposure to DMF. The electrophoretic fractionation of shed cell surface protein from HCT MOSER cells and HCT MOSER cells treated with DMF for 14 and 21 days is depicted in Chart 4A, 4B, and 4C, respectively. Although there was a progressive decrease of csp120 on HCT MOSER cells with increased time of exposure to DMF, all 3 shed cell surface protein profiles exhibit a prominent amount of ^{125}I-labeled material with a molecular weight of ∿120,000. Curiously, while HCT MOSER cells had a large amount of csp180 (Chart 1A) the shed cell surface protein from these cells (Chart 4A) contained little, if any, protein of 180,000 molecular weight. However, HCT MOSER cells grown in DMF shed a substantial quantity of 180,000 molecular weight surface protein the amount of which was proportional to time of growth in DMF (Chart 4B and 4C).

To determine if HCT MOSER cells grown in the presence and absence of DMF released fibronectin into culture medium, conditioned medium from these cells was collected, electrophoresed, and transferred to nitrocellulose sheets which were incubated with antisera and ^{125}I-protein A as described. An amount of material from an equal number of cells (based on DNA assay) was loaded in each lane. There was no detectable amount of material immunoreactive with anti-fibronectin in the medium in which HCT MOSER cells were grown (Fig. 10B).

Chart 4 - Shed cell surface proteins from HCT MOSER cells (A), HCT MOSER cells grown in 0.5% DMF for 14 days (B), and HCT MOSER cells grown in 0.5% DMF for 21 days (C). Arrows in panels B and C indicate the 180,000 molecular weight shed surface protein which was increased as a function of time of exposure of HCT MOSER cells to DMF.


A
^{125}I CPM (x10^{-3})
MOLECULAR WEIGHT (x10^{-3})
200
116
92
66
45
31


B
^{125}I CPM (x10^{-3})
MOLECULAR WEIGHT (x10^{-3})
200
116
92
66
45
31

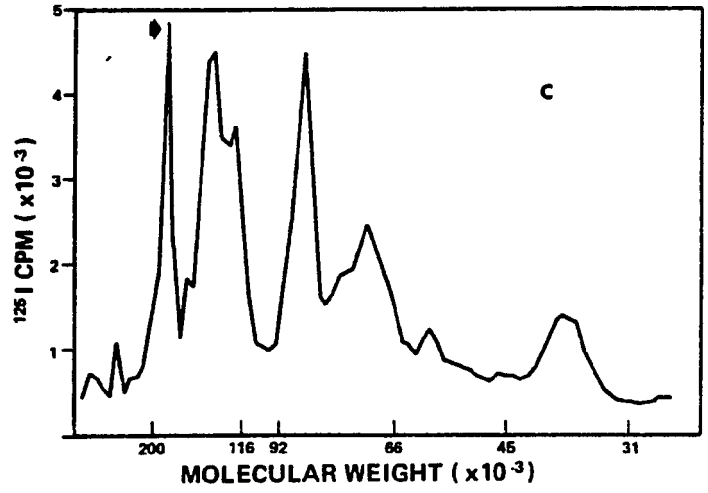
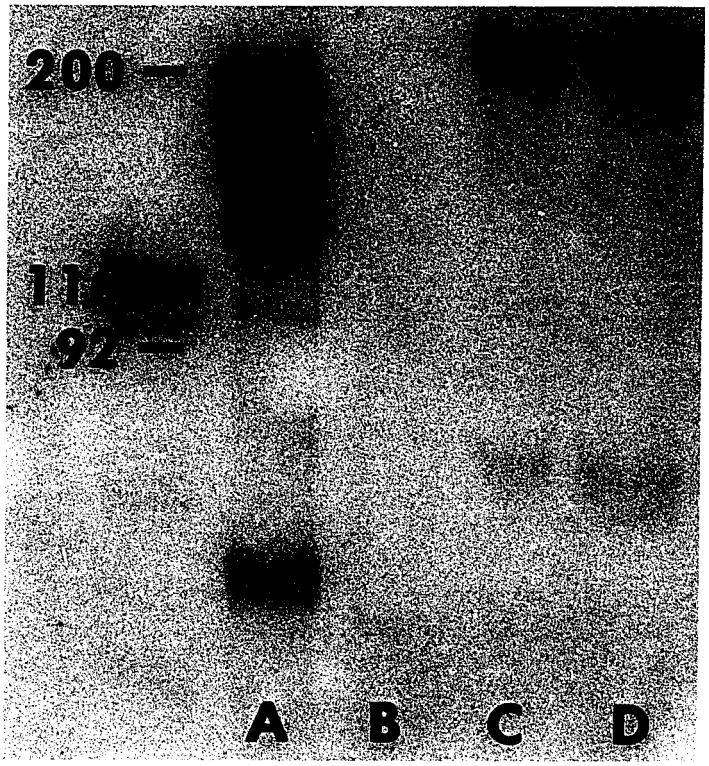

C
^{125}I CPM (x10^{-3})
MOLECULAR WEIGHT (x10^{-3})
200
116
92
66
45
31

Figure 10 - Detection of anti-human fibronectin immunoreactive material released from HCT MOSER cells (B), HCT MOSER cells grown in the presence of 0.5% DMF for 14 days (C), and HCT MOSER cells grown in 0.5% DMF-supplemented medium for 21 days (D). Human plasma fibronectin was run in parallel as a control for anti-fibronectin activity (A). Conditioned medium from cells grown in the presence and absence of DMF and plasma fibronectin were electrophoresed, transferred to nitrocellulose membrane, and incubated in anti-fibronectin and ^{125}I-labeled protein A as described in "Materials and Methods." Molecular weights are as indicated (x 10^{-3}).


200
92
A
B
C
D

This is in marked contrast to DMF-treated HCT MOSER cells which clearly synthesized and released into medium material reactive with anti-fibronectin (Fig. 10C and 10D). The molecular weight of the immunoreactive material from DMF-treated HCT MOSER cells approximates that of cellular fibronectin which is 220,000 ± 20,000.

To control for binding of fibronectin contained in FBS to HCT MOSER cells and its later release into serum-free medium, FBS was assayed for material reactive with anti-human fibronectin. To this end, FBS was electrophoresed and transferred onto nitrocellulose sheets as was conditioned medium from DMF-treated HCT MOSER cells. The nitrocellulose sheets were then incubated under anti-human fibronectin and ^{125}I-labeled protein A as described in "Materials and Methods." FBS was found to contain no detectable material immunoreactive with anti-human fibronectin (data not shown).

Cellular viabilities, as determined by trypan blue exclusion, for HCT MOSER cultures after radioiodination ranged between 90% and 95%. Viabilities of HCT MOSER cultures subjected to DMF treatment varied between 81% and 89%.

DISCUSSION

Application of DMF to culture medium in which the spontaneously arising human colon cancer line HCT MOSER was grown resulted in cell surface alterations which were coincident with cell morphology changes. The cell surface and morphology changes were reversible as well as dependent upon time of exposure to DMF and DMF concentration. DMF has been noted to reversibly produce decreased tumorigenicity and clonogenicity in human colon cancer cells (30,87) and to reversibly modify expression of human colon cancer cell tumor-related markers (30,37). These findings indicate that in vitro exposure of human colon carcinoma cells to DMF induces a more benign, better differentiated phenotype. Thus, the cell surface response of HCT MOSER cells to DMF may be associated with induced differentiation.

The AKR-2B/AKR-MCA cell lines were used as a model system to initially investigate and document any cell surface manifestations related to DMF-induced differentiation before examining the effects of DMF on a transformed cell line for which there is no nontransformed counterpart for comparison,

e.g., HCT MOSER. Despite the species and cell type differences, there are parallels between the cell surface effects of DMF on HCT MOSER cells and the previously described actions on AKR-MCA methylcholanthrene-transformed mouse fibroblasts (61). Both cell lines exhibited a surface protein of 85,000 - 87,000 molecular weight which was decreased upon DMF treatment. The amounts of cell surface proteins with molecular weights less than 200,000 tended to be diminished when HCT MOSER and AKR-MCA cells were exposed to DMF. Other similarities between the two cell lines and their response to DMF include: synthesis of TGF (54,65); DMF-produced cell surface effects as a function of time of exposure to DMF and DMF concentration; similar cell and culture morphologies following DMF treatment; and DMF-enhanced production of cell surface-associated material immunoreactive with anti-fibronectin.

The possibility that the anti-fibronectin immunoreactive material on the surfaces of DMF-treated AKR-MCA and HCT MOSER cells and released from DMF-treated HCT MOSER cells actually is fibronectin is potentially important. Fibronectin is related to cellular differentiation (1,44-46, 79), control of cell replication (11), transformation (26), regulation of gene expression (85), and modulation of invasiveness and metastasis of malignant cells (78,92). The effect of DMF on HCT MOSER and AKR-MCA cell morphology is consistent with expression of fibronectin as fibronectin promotes cell attachment and spreading and is involved in cell

morphology (see Refs. 45,46,78,79 for reviews). Furthermore, the anti-fibronectin reactive material on the surfaces of DMF-treated AKR-MCA cells exhibited a fibrillar arrangement compatible with the appearance of fibronectin matrix on non-transformed cells (45,78,79). This may be regarded as supporting the contention that the DMF-induced surface material on AKR-MCA cells reactive with anti-fibronectin is indeed fibronectin. Expression of fibronectin by AKR-MCA cells after exposure to a differentiation-inducing agent is certainly expected since the AKR-MCA cell line originated from non-transformed fibroblasts which normally manifest surface fibronectin (see Refs. 44-46 for reviews). Thus, the induced presence of an anti-fibronectin immunoreactive surface material on AKR-MCA cells correlates with the maturational properties of DMF. Low-level synthesis of fibronectin by intestinal epithelial cells in vitro has been reported (see Ref. 46 for review). Therefore, as with AKR-MCA cells, DMF-enhanced synthesis of fibronectin by HCT MOSER would be anticipated if there were assumption of a more differentiated phenotype. Two observations indicate that fibroblast contamination was not responsible for production of anti-fibronectin immunoreactive material by DMF-treated HCT MOSER cultures: (1) there was continued elaboration of CEA by HCT MOSER cells after exposure to DMF; and (2) removal of DMF from HCT MOSER cells resulted in reversion to a pre-DMF phenotype. Hence, those cells of DMF-treated HCT MOSER cultures apparently possessed certain characteristics of colon cancer cells which should not be displayed by fibroblasts.

Unlike AKR-MCA cells subjected to DMF, the pattern of material reactive with anti-fibronectin on the surfaces of DMF-treated HCT MOSER cells was diffuse rather than fibrillar. Also, ^{125}I incorporation data imply that there was no buildup over time of ∿200,000 molecular weight surface protein on HCT MOSER cells secondary to DMF treatment as was previously shown for DMF-treated AKR-MCA cells (61). However, csp200 was essentially the only surface protein available for radioiodination on HCT MOSER cells grown in DMF for 21 days. If csp200 is the same as anti-fibronectin immunoreactive material then this surface component may be only transiently associated with the cell surface before being released from the cell. Inability to retain the anti-fibronectin material on the surface may account for lack of a well defined fibrillar pattern on DMF-treated HCT MOSER cells.

Assuming that the anti-fibronectin immunoreactive material is actually fibronectin there are explanations why this material did not accumulate on HCT MOSER cells grown in DMF. Hynes and Yamada (46) and Ruoslahti *et al*. (78,79) have speculated that interaction between fibronectin and other matrix constituents is involved in the arrangement of cellular fibronectin as a fibrillar extracellular matrix. Thus, DMF-treated HCT MOSER cells may have been devoid of the normal complement of matrix components necessary for retention of fibronectin. Another possibility is that there was alteration of the carbohydrate moieties of cell surface membrane glycoconjugates. This would impair interaction with fibronectin and result in decreased binding or attachment (8,79).

Alternatively, modification of fibronectin may interfere with its incorporation into a cellular matrix. Zerlauth and Wolf (110,111) have hypothesized that changes in the fibronectin molecule resulting from phosphorylation/dephosphorylation or glycosylation processes might be the direct cause for increased shedding of fibronectin from transformed human lung fibroblasts. According to Zerlauth and Wolf (111), modified fibronectin is not identified at the cell surface as fibronectin and is therefore not integrated into the pericellular matrix. It has been shown that retinoic acid (a differentiation agent) can affect glycosylation of fibronectin produced by normal and malignant cells (8,63). Bernard et al. (8) and Meromsky and Lotan (63) noted that modified surface attachment of fibronectin accompanied retinoic acid-altered glycosylation of fibronectin in their cell systems. Although altered glycosylation of fibronectin may inhibit its binding to the cell surface, carbohydrate on fibronectin has not been demonstrated to be involved in the biological functions of fibronectin (46). So, if DMF-induced fibronectin from HCT MOSER cells did undergo altered glycosylation it would continue to exert the same biological activity as normal fibronectin.

It was observed during this study that trypsin-treatment of HCT MOSER cells grown in DMF for 14 days caused the cells to exhibit a pre-DMF morphology and cell surface profile. This suggests that the effects of DMF on HCT MOSER cells were mediated by a cell surface-associated protein

which required a relatively long amount of time (2-3 weeks) to accumulate and to manifest its actions. DMF-induced fibronectin could produce the phenotypic response of HCT MOSER cells to DMF via interaction with the extracellular matrix as the extracellular matrix functions in the regulation of gene expression and differentiation (10,35,40,96,99). In this scenario, cells capable of producing fibronectin could promote differentiation in cells unable to synthesize fibronectin but which are proximate to the cells which do.

We have previously suggested that the AKR-2B/AKR-MCA surface proteins of molecular weight less than 200,000 which are increased upon transformation and eliminated upon DMF treatment may be fibronectin fragments (52,61). Such a proposal is consistent with observations and proposals made by others which relate to proteolytic degradation of fibronectin in transformed cells (20,44,46,70). This postulate is significant because certain proteases have been shown to be associated with transformation (20,68) and fibronectin fragments have been shown to enhance transformation (26). However, if the csp85 and csp63 from AKR-MCA cells and the csp180 and csp120 from HCT MOSER cells are fibronectin fragments, they lack an epitope reactive with the antisera to fibronectin used in this study.

CONCLUSIONS

The research described in this dissertation has produced information upon which the following conclusions have been made. These conclusions also serve to summarize the findings presented herein.

1. Exposure of normal AKR-2B fibroblasts to TGF produced transient cell surface changes which resembled those permanent surface alterations associated with methylcholanthrene-induced transformation.
2. TGF and methylcholanthrene produced a decrease in 200,000-250,000 molecular weight surface protein and increase of surface proteins with molecular weights of 85,000 and 63,000.
3. The surface effects described above were not related to mitogenesis.
4. Growth of transformed AKR-MCA fibroblasts and HCT MOSER colon cancer cells in DMF resulted in marked changes in cell morphology.
5. In both AKR-MCA and HCT MOSER cell lines the morphology

changes caused by DMF were accompanied by an increase in cell surface protein of 200,000-250,000 molecular weight.

6. Coincident with the DMF-induced increase in ^{125}I-labeled surface protein of 200,000-250,000 molecular weight on AKR-MCA and HCT MOSER cells was expression of cell surface material immunoreactive with anti-fibronectin.

7. ^{125}I incorporation data implied that the 200,000 - 250,000 molecular weight surface protein accumulated on DMF-treated AKR-MCA cells but not on HCT MOSER cells exposed to DMF.

8. Induction of cell surface fibronectin by DMF treatment would not be surprising for AKR-MCA cells since these cells are transformed fibroblasts. However, expression of fibronectin by DMF-treated HCT MOSER cells does seem unusual. Contamination of the HCT MOSER cell line by fibroblasts was considered as being responsible for production of anti-fibronectin immunoreactive material after exposure of HCT MOSER cells to DMF. This possibility was eliminated by demonstrating that DMF-treated HCT MOSER cells exhibited CEA.

9. It appeared that most of the anti-fibronectin immunoreactive material induced by DMF treatment of HCT MOSER cells was released into the medium and was only transiently associated with the cell surface.

10. Possible reasons for decreased retention of fibronectin on HCT MOSER cells include absence or modification of

surface components which interact with fibronectin and alteration of the fibronectin molecule.

11. In the HCT MOSER system, DMF may produce a more benign or differentiated phenotype via promotion of low level synthesis of fibronectin.

12. Additional cell surface changes produced by DMF were decrease of 85,000 molecular weight protein on AKR-MCA cells and decrease of 120,000 and 180,000 molecular weight material on HCT MOSER cells.

13. All of the DMF-promoted cell surface effects noted above were dependent on length of DMF treatment and DMF concentration.

14. DMF was also determined to be capable of preventing transformation of AKR-2B cells by TGF.

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