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EVIDENCE OF A PHOSPHORYLATED PROTEIN SPECIFIC OF HUMAN COLON CARCINOMA CELLS

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Summary : A phosphorylated 42 kilodalton protein has been detected in HT29 and Caco2, two colonic tumor cell lines, by immunoprecipitation technique. The addition of a differentiating agent such as retinoic acid to the culture medium of the different cell lines induces a 50 % reduction of the cellular growth but the 42 K protein remains present.

INTRODUCTION

The antigenic pattern of a human colonic tumor cell line (HT29) has been studied by immunofluorescence technique (1). It has been shown that this cell line kept the ability to synthesize three colon tumor antigens : the carcinoembryonic antigen (CEA), the nonspecific cross reacting antigen (NCA) and the membrane associated autoantigen (MTA). In this paper we follow by immunoprecipitation technique the protein production of HT29 cells in culture and demonstrate the synthesis of a specific phosphorylated 42 kilodalton (42 K) protein. The possible reversibility of tumor growth and the cellular differentiation of some tumor cells under the effect of various chemical agents have been described (2-7). The existence of tumor cell markers is an important tool in the understanding of the mechanism of normal or neoplasic cellular growth and differentiation. Therefore the effect of a differentiating agent, retinoic acid, on the cellular growth and the production of the 42 K protein of HT29 but also CaCo2, a colorectal carcinoma cell line and MDA MB231, an established mammary tumor cell line, was followed.

MATERIALS AND METHODS

Cells and culture conditions : The human colorectal carcinoma cell lines HT29 and Caco 2 were kindly supplied by Dr. J. Fogh (Sloan Kettering Institute, Rye, N.Y.). The mammary tumor cells MDA-MB-231 were from Dr. E.M. Jensen (Mason Research Institute Rockville, Ma). HT29 and MDA-MB 231 cells were cultivated with RPMI 1640

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nutrient medium (Gibco) supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml, gentamycin 50 µg/ml) fresh glutamine 2 mM (Gibco) and 10 % (V/V) fetal calf serum (Flow Laboratory). Caco 2 were grown in Eagle's minimum essential medium with Earle's and non essential aminoacids (Eurobio) supplemented with glutamine and antibiotics as above plus 15 % (V/V) fetal calf serum. The initial cell populations were about 1 to 5 x 10^5 cells/ml. The cultures were incubated at 37° in an atmosphere of CO2 : air (5 : 95 %) in presence (HT29 + Re ; Caco 2 + Re ; MDA + Re) or absence of 3.10^{-5}M 1,3 cis-retinoic acid (Roche). The three cell lines were consistently mycoplasma free.

Antisera : Anti HT29 serum was prepared by repeated intravenous injections of whole HT29 cells. Anti normal adult colon serum was obtained by intradermal injections of soluble colon extract emulsified in complete Freund's adjuvant. Anti HT29 serum, anti normal adult colon serum as well as the control normal rabbit serum were successively absorbed with lyophilized human normal plasma, then by A and O human red blood cells. An aliquot of the anti HT29 serum so obtained was also absorbed by actin, the amount of actin used absorbing completely anti-actin serum.

Immunoprecipitation, electrophoresis and detection of labelled proteins : According to the technique described by Schwyzer (8) the different cell lines were seeded at 5 to 10 x 10^6 cells per 10 cm petri dish (Corning). 3 days after seeding the cultures were incubated for 1 hour in methionine (or phosphate) free Eagle's minimal essential medium and then labeled for 3 hours either with 25 to 50 µCi of L - (^{35}S) methionine per ml (845 to 1230 Ci/mmol) or with 100 µCi of $^{32}PO_4$ per ml (both radiolabeled compounds from CEA, France) in methionine (or phosphate) free medium. After labeling, the cells were scraped, washed twice with ice cold phosphate buffered saline and suspended at a concentration of 2 to 5 x 10^7 cells per ml Tris-buffered saline containing 1 % Nonidet P-40 (Sigma) 10 % glycerol (Merck), 10^{-3} M diisopropylfluorophosphate (Serva), 0,5 % triton X 100 (Intertechnique) 0,5 % sodium deoxycholate (Fluka). After 20 min shaking at 4°, the extract was centrifuged for 30 min at 20 000 x g. Aliquots of the supernatant were then incubated with 5 µl of the various antisera or the control rabbit serum and 20 µl of settled protein A-Sepharose Cl-4B (Pharmacia). The suspension was gently agitated at least 3 hours at 4° then poured into a column. The Sepharose was washed with 4 times 0.5 ml of 0.1 M Tris HCL pH 9 containing 0.5 M LiCl and 1 % 2-mercaptoethanol. Immune complexes were then eluted with 3 sepharose volumes of 0.06 M Tris HCl (pH 6.7),15 % glycerol, 2 % sodium dodecylsulfate (SDS), 5 % 2-mercaptoethanol and 0.001 % bromophenol blue. After boiling the eluates for 3 min at 100°C the separation of the antigens from immunoglobulin G was performed by polyacrylamide gel electrophoresis in the presence of 0.1 % SDS as described by Laemmli (9); polyacrylamide gels were 12.5 %, 10 % or 7,50 % . Electrophoresis was carried out for 4 hours at 80 V. The gels were stained with Coomassie brilliant blue R (Sigma), destained, dried and exposed either to Fuji X-Ray films or Kodak X-Omat AR films.

RESULTS

Figure 1 shows the autoradiograms of SDS polyacrylamide gel separation of the immunoprecipitable proteins of HT29 labeled cells. When we compare the response of the cells to normal rabbit serum (tracks 1,3,5) or to HT29 total cells antiserum (tracks 2, 4,6) we observed, when the cells are labeled with (^{35}S) methio-

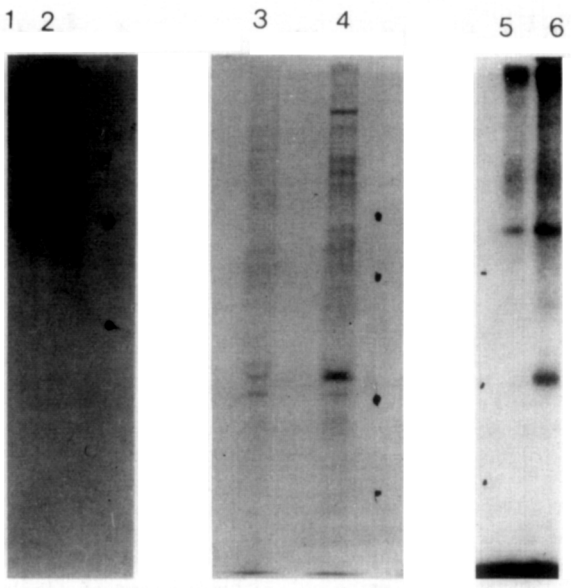

Figure 1 : SDS-polyacrylamide gel autoradiograms of labeled immunoprecipitated proteins from HT29 cell line. The cells were labeled with (^{35}S) methionine (tracks 1 to 4) or with $^{32}PO_4$ (tracks 5-6). The gels contained 12.5 % (tracks 1,2) or 7.5 % (tracks 3 to 6) polyacrylamide. The sera were normal rabbit serum (tracks 1,3,5) and anti HT29 (tracks 2,4,6). The dots were protein standards : from top to bottom phosphorylase b (mol. wt 94,000) bovine serum albumin (67,000), ovalbumine (45,000), chymotrypsinogen A (25,000).

nine, a 42 K to 45 K protein immunoprecipitable by anti HT29 (tracks 2 and 4) and not by normal rabbit serum (tracks 1 and 3). Moreover this 42-45 K protein is phosphorylated (track 6) as shown when we use cells labeled with $^{32}PO_4$ (tracks 5 and 6).

The 42-45 K protein is phosphorylated by HT29, HT29 + Re, Caco 2 and Caco 2 + Re (tracks 2,5,8,11 of figure 2) but apparently not by MDA and MDA + Re (tracks 14 and 17 of figure 2). It is to be noticed that the 42-45 K protein of HT29 or Caco 2, treated or not with retinoic acid, which migrates on polyacrylamide gels like actin (42 000 daltons) is not precipitated by anti normal colon serum (tracks 3,6,9,12 of figure 2).

After 3 days of culture in presence or absence of retinoic acid (3.10^{-5}M) HT29, Caco 2 and MDA cells were counted. In presence of retinoic acid the cellular growth of the three treated cell lines was repeatedly reduced by 50 %. Labeled with (^{35}S) methionine (figure 3 and 4) aliquots of the various cellular lysates are immunoprecipitated with different antisera. As previously described in presence of anti HT29 (tracks 2,6,10,14,18,

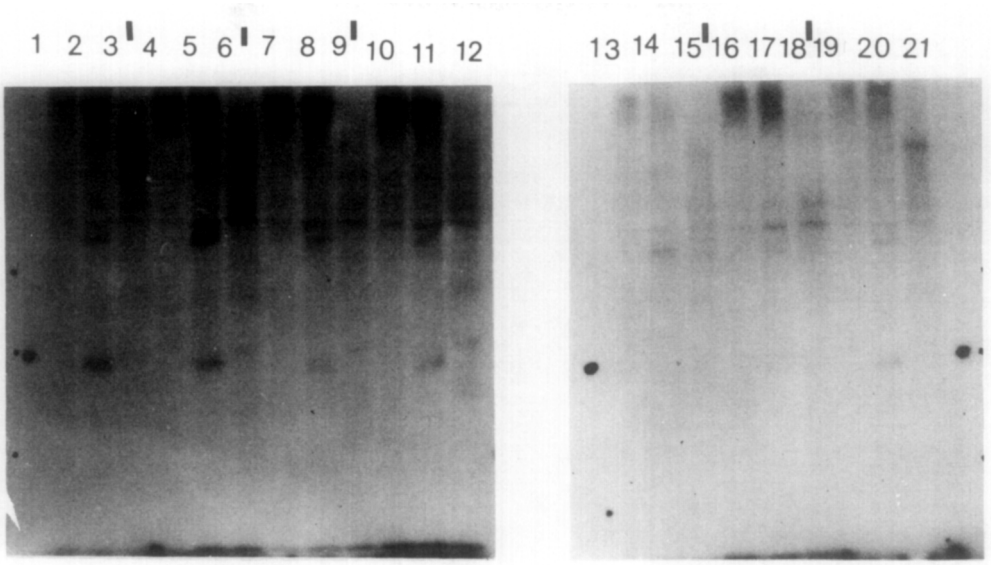

Figure 2 : SDS-polyacrylamide gel autoradiograms of labeled immunoprecipitated proteins from three cell lines cultivated in presence or absence of retinoic acid (3.10^{-5}M). The cells were labeled with $^{32}PO_4$. The 2 gels were 10 % in polyacrylamide. The cells were HT29 (tracks 1 to 3), HT29 + Re (tracks 4 to 6), Caco 2 (tracks 7 to 9) Caco 2 + Re (tracks 10 to 12), MDA (tracks 13 to 15), MDA + Re (tracks 16 to 18), HT29 (tracks 19 to 21). The sera were normal rabbit serum (tracks 1,4,7,10,13,16,19) anti HT29 serum (tracks 2,5,8,11,14,17,20) and anti normal adult colon serum (tracks 3,6,9,12,15,18,21). The protein standards were from top to bottom : bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (25,000). The single dot corresponds to the migration of actin (42,000).

22) a protein of 42 to 45 K, with the mobility of actin (42 K) is precipitated. Anti HT29 absorbed with actin immunoprecipitates also the 42-45 K protein (tracks 3,7,11,15,19,23) while normal rabbit serum does not. Normal adult colon antiserum also does not immunoprecipitate the 42-45 K protein but a 28 to 30 K protein is clearly detected in HT29 and MDA cells treated or not with retinoic acid (tracks 4,8,12,16 of figures 3 and 4).

Labeled with $^{32}PO_4$, HT29 proteins (figure 5) were immunoprecipitated by the antisera previously used. Gels containing either 10 % (tracks 1 to 4) or 7.5 % (tracks 5 to 8) polyacrylamide were run in parallel. The autoradiograms of these gels show that anti HT29 and anti HT29 absorbed by actin precipitate a 42000 dalton phosphorylated protein (tracks 2,3,6,7) while the normal rabbit serum or the anti normal adult colon serum does not, (tracks 1, 4, 5, 8).

DISCUSSION

After labeling the cells with (^{35}S) methionine, HT29 and CaCo2, two different human colonic tumor cell lines, but also

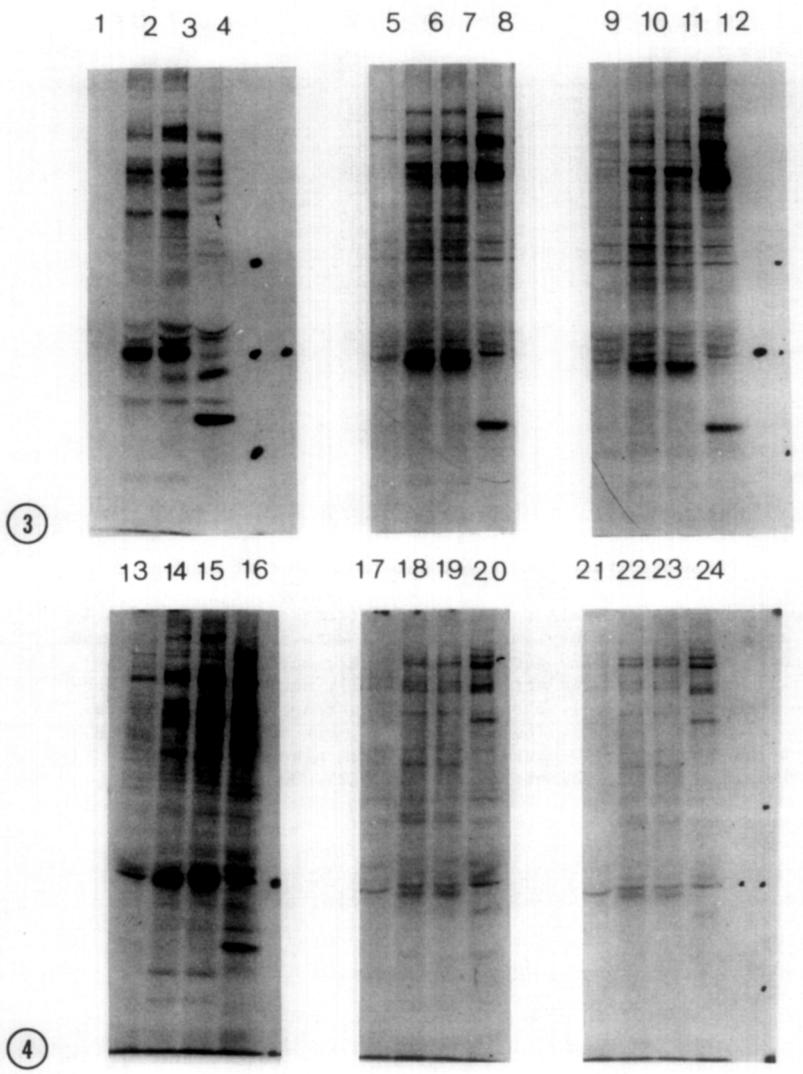

Figure 3 and 4 : SDS-polyacrylamide gel autoradiograms of labeled immunoprecipitated proteins from three cell lines cultivated in presence or absence of retinoic acid (3.10^{-5}M). The cell were labeled with (^{35}S) methionine. The 2 gels (tracks 1 to 12 and 13 to 24) contained 10 % polyacrylamide. The cells were HT29 (tracks 1 to 4), MDA (tracks 5 to 8), MDA + Re (tracks 9 to 12), HT29 + Re (tracks 13 to 16) Caco 2 (tracks 17 to 20) Caco2 + Re (tracks 21 to 24). The sera were normal rabbit serum (tracks 1, 5,9,13,17,21), anti HT29 serum (tracks 2,6,10,14,18,22), anti HT29 serum absorbed with lyophilized actin (tracks 3,7,11,15,19, 23), anti normal adult colon serum (tracks 4,8,12,16,20,22,24). The protein standards were from top to bottom : bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen A (25,000). The single dot corresponds to the migration of actin (42,000).

the mammary tumor cells MDA MB 231 synthesize a protein of 42 K clearly detectable by immunoprecipitation with HT29 total cell antiserum. The fact that anti HT29 after being absorbed with

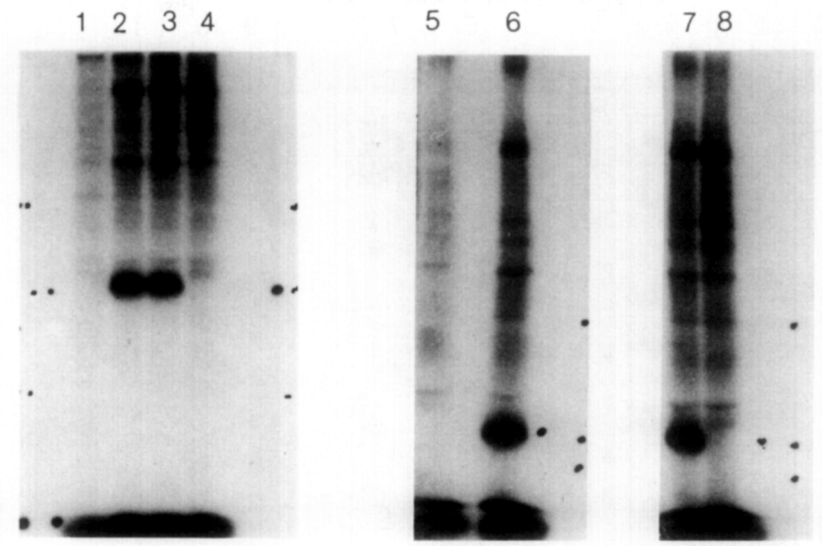

Figure 5 : SDS-polyacrylamide gel autoradiograms of labeled immunoprecipitated proteins from HT29 cell line. The cells were labeled with $^{32}PO_4$. The gels contained 10 % (tracks 1 to 4) or 7,5 % (tracks 5 to 8) polyacrylamide. The sera were normal rabbit serum (tracks 1,5), anti HT29 serum (tracks 2,6), anti HT29 serum absorbed with lyophilized actin (tracks 3,7), anti normal adult colon serum (tracks 4,8). The single dot corresponds to the migration of actin (42,000). The aligned dots correspond to standard proteins : from top to bottom bovine serum albumin (67,000), ovalbumin (45,000), chymotrypsinogen A, (25,000).

actin (molecular weight 42 000) still precipitates the protein eliminates the possibility that the 42 K protein is identical to actin. Moreover the 42 K protein is phosphorylated (figure 1,2 and 5) in the case of HT29 and Caco2. This protein is not detectable by anti normal adult colon serum which led us to the hypothesis that it could be specific of the colon carcinoma cell lines studied. The 42 K protein is still present when the various cell lines are treated with retinoic acid. This suggests that the differentiating agent used does not alter the expression of this antigen in the conditions of culture tested. However it is to be noticed that the growth of the three cell lines is strongly inhibited.

In conclusion if the 42 K protein is a potential useful marker of the human colon carcinoma cells, its role and importance is still to be defined. The direct chemical characterization and peptide mapping of the 42 K phosphorylated protein could be of help to elucidate the significance of this antigen as well as its analyse in various types of epithelial tumor cells and in selected conditions of culture (10).

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