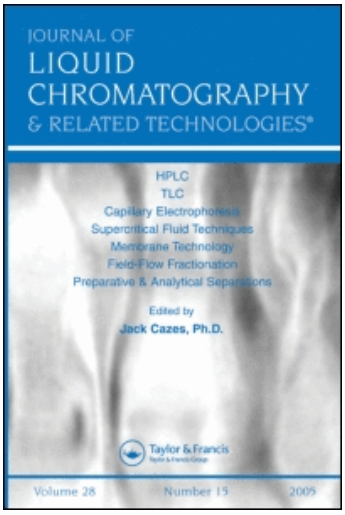

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HYPOTHESIS ON DIFFERENTIATION OF CANCEROUS CELLS: A POSSIBLE MEANS FOR ISOLATION BY HPLC OF DIFFERENTIATION FACTORS FROM MOUSE EMBRYO CELLS

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ABSTRACT

The cancerous cells express particular genes, which are normally active during embryonic development. On the other hand, young embryos inoculated with teratocarcinoma or leukaemia cells are able to differentiate them and to regulate their division rate, by producing differentiation factors.

A means of partial purification of these factors, extracted from mouse embryo cell nuclei, and separated by high performance liquid chromatography on anion-exchange column, is described. The possibility of treatment of cancerous cells by this way is discussed.

EMBRYONIC CHARACTER OF CANCEROUS CELLS

The expression of the genes of the cancerous cells is profundly modified, compared to that of the normal cells. The levels of 12% of the proteins of the SV 40 transformed cells are altered 2 fold or more by viral transformation (1). In particular, a lot of normal antigens are present, which appear usually during embryonic development.

The liver α foeto protein of the fetal liver is found in the liver tumors (2). The carcinoembryonic antigen normally present in the fetal

colon is also produced by cancerous colon cells (3). A placentary hormone is secreted by certain human lung tumors (4). Many oncogenes are maximally expressed in the mouse embryo : c ras at the 7^{th} day, c abl around the 12^{th} day, c fos before the 8^{th} day (5). The human oncogene c amv is active in all immature myeloid and lymphoid T cells, but not in mature B and T cells, and is only slightly expressed in leukaemic cells differentiated by dimethylsulfoxide (DMSO) or retinoic acid (6). There is evidence of homologies between certain oncogenes (myc, int_1, v rel, src) and homeotic genes of insects involved in larval development (7).

Is it reasonable to generalize these results to all the oncogenes? In other words, is an early expression a necessary condition for a gene to be an oncogene? The question is left open, but this hypothesis is attractive.

Conversely, certain genes active in the embryo are oncogenic : when dispersed embryo cells are grafted on adult organisms or set on tissue culture, they divide without regulation and form special tumors called teratocarcinomas (8). In this way, tumor formation does not need any mutation or presence of an active promotor near some particular gene. The mere disorganization of the embryo structure is sufficient to induce carcinogenesis.

DIFFERENTIATION OF CANCEROUS CELLS

The first example was given by C. Friend in 1971, who differentiated erythroblast leukaemic mouse cells in culture with DMSO (9). Many chemicals have been tested since then (bacterial lipopolysaccharides, insulin, phorbol esters, plant lectins, vitamin A derivatives such as 13 cis retinoic acid, etc...) (10). Unfortunately these treatments, which induce differentiation of some cancerous clones, are without effect on others.

The most clear cut example of the differentiation of malignant cells is that of teratocarcinoma cells, which contribute to the embryo development when injected into a blastocyst cavity, giving normal cells of all of the tissues of the adult (11-14). One blastocyst can regulate one carcinoma cell consistently. However, the results are less conclusive when several cells are injected together or when B 16 melanoma cells are used (13). Similarly, myeloid leukaemic cells injected into 10 days old mouse embryos, lose their malignant character and the apparently healthy adult mice, have granulocytes containing a marker derived from the cancerous cells (15).

The regulating capacity towards myeloid leukaemic cells decreases with age and disappears after the 11^{th} day in the mouse embryo (15). Thus it seems that differentiation factors, probably proteins, are present in the young embryo and are able to transform cancerous cells into normal, non pathogenic, differentiated ones. The formation of teratocarcinomas from dissociated embryo cells grafted on adult organisms is likely to be due to depletion by dilution of these differentiation factors. The genes which were expressed remain perpetually active, especially the oncogenes, which are responsible for the malignant character.

Many experiments have shown that diffusible substances, including growth factors (CSF_1, G-CSF, GM-CSF, IL_3) and differentiation inducers (MGI_2) produced by fibroblast cultures are able to differentiate clones of myeloid leukaemic cells (16-19). Treatment by these products extends the survival of mice injected with cancerous cells. However the results depend on the clone of malignant cells used. Some clones differentiate easily and stop dividing, others are less sensitive and need combined treatment with chemicals or do not differentiate at all. This limitation could be attributed to the fact that the growth and differentiation factors used in these experiments were produced by one particular kind of already differentiated cells (fibroblasts) which were not necessarily active on the expression of all the oncogenes. Moreover, differentiation factors produced by adult tissues are ineffective towards injected cancerous cells since myeloid leukaemic cells or teratocarcinoma cells kill adult mice within some weeks. In the latter case, some cells differentiate (teratoma) while the other ones are unaffected and divide rapidly (carcinoma), likely by lack of adequate differentiation inducers.

The results of the afore mentioned experiments suggest that the differentiation factors produced by immature cells could be an efficace treatment for differentiating cancer cells. For this reason, we have tried to extract them from mouse embryos. These factors are probably mainly localized in the nucleus, since they work by DNA binding (20). The separation of the nuclei has the advantage of getting rid of the cytoplasmic proteins. After disruption of the nuclei, the different nuclear proteins can be separated by HPLC. Another way would be to extract all the proteins of the cells and to separate the DNA binding proteins by affinity chromatography on a DNA-bonded column, but as the conditions of binding of the differentiation factors are not known (pH, molarity), they could be eluted during the washing of the column.

MATERIALS AND METHODS

Nuclei from mouse embryos were prepared by a modification of the method of Widnell and Tata (21,22). The tissues were homogenized in five volumes of 0.25 M sucrose, 3 mM $CaCl_2$ (A), in a glass-Teflon homogenizer, using eight up and down strokes at 2000 rpm. After filtration through three layers of gauze, the homogenate was centrifuged at 1000 g for 10 minutes. The pellet was then homogenized in 2.4 M sucrose, 3 mM $CaCl_2$ and centrifuged at 40 000 g for 1 hour. The supernatant was discarded and the pellet nuclei were washed with A and collected by centrifugation at 1000 g for 10 minutes. The nuclei were resuspended in the same medium containing 1% (V/V) Triton X 100, for removing the inner and outer nuclear membranes. The Triton X 100 was removed by washing the nuclei twice with A. The purified nuclei were resuspended in Tris 1 mM pH 8, 0.1 mM EDTA and sheared for 1 minute in a Virtis homogenizer (23). The suspension was then centrifuged 30 minutes at 15 000 g to remove any insoluble material. All operations were performed at 4°C. Glass-Teflon and Virtis homogenizers were chilled with ice.

The supernatant was fractionated by HPLC on an anion-exchange DEAE 5 PW column (0.75 x 7.5 cm) by using a linear NaCl gradient (0 to 1 M in 30 minutes) in 0.02 M Tris buffer pH 8, at a flow rate of 1 ml/minute. Column efluent was monitored spectrophotometrically at 280 nm.

The HPLC apparatus was a Waters liquid chromatograph (M 510 pumps, U6K injector, 490 E detector). The peak fractions were pooled and concentrated in an Amicon ultrafiltration apparatus containing a PM 10 membrane. They were washed two fold with 5 ml 9‰ NaCl and stored at -20°C.

RESULTS AND DISCUSSION

The chromatograms presented between 15 and 20 partially or completely resolved peaks (Fig.1 and Fig.2). The same pattern was obtained by repetition in the same conditions. All the pooled fractions had a protein absorption spectrum ($\frac{280\ nm}{260\ nm}$ ratio between 1.2 and 1.7), except the last preponderant peak which was constituted by nucleic acid ($\frac{280\ nm}{260\ nm}$ ratio = 0.5).

The experiments were performed on 5, 10 and 15 days old mouse embryos. In the first case, contamination by uterus tissues could not be avoided.

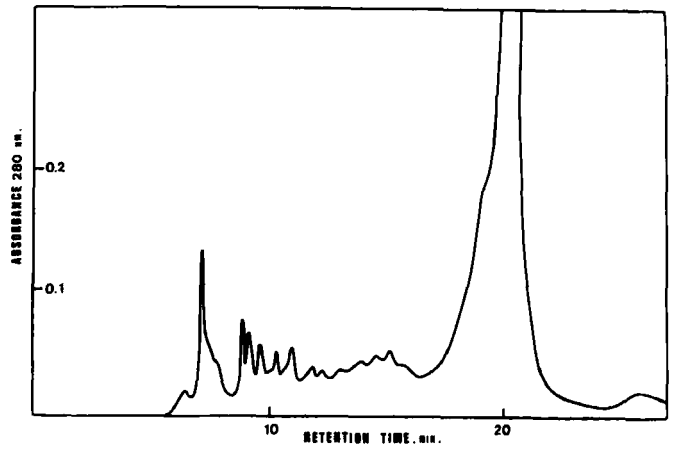

Fig.1 : Anion exchange HPLC of nuclear proteins extracted from 10 days old mouse embryos (17g). Conditions described in the text.

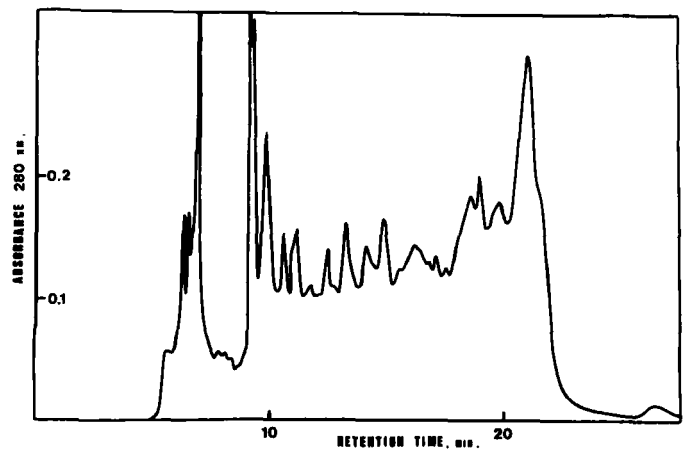

Fig.2 : idem Fig.1, with 15 days old mouse embryos (23g).

The comparison between 10 and 15 days embryo nuclear proteins showed that the quantity of proteins compared to that of nucleic acid increased with age. In the three experiments, several peaks seemed to be identical, on the basis of their retention time, but in different proportions.

No further characterization has been performed in this preliminary work. The differentiation factors are probably in so minute concentration that they may be hidden by other proteins like histones. These nuclear protein fractions have now to be tested for their differentiation capacity on cancerous cells, for instance using the protocols described by L. Sachs (19) with leukaemic cells in culture, or by comparing the survival of mice inoculated with leukaemic cells.

It is possible that it would not be necessary to separate and purify extensively each differentiation factor in order to test their biological activity. Perhaps it would be better to have a mixture of factors coming from different immature tissues and acting on the expression of several oncogenes. Indeed, a cell becomes cancerous only after multiple mutations and modifications of the expression of its genes and is always evoluting during the course of malignancy.

There is a great similarity between the corresponding oncogenes of different species (human, cat, rat, chicken and insects) (24). The src gene from chicken hybridize with all vertebrate DNA and also with that of Drosophila. Between human and chicken, the differences in sequence is only 15%. The ras gene from rat hybridize also with yeast DNA. It is conceivable that the differentiation inducers, which act on their expression, could be also highly conserved during evolution and then it would be possible to differentiate cancerous cells with factors from another species.

Finally, as differentiation factors produced by differentiated cells are inactive towards carcinoma cells or certain clones of leukaemic cells, it is likely that those extracted from embryonary cells would have no effect on normal differentiated cells. Those that might be active on normal immature cells (as myeloid precursors or germ cells) could be discarded by chromatographic separation.

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