ISOENZYME PATTERN AND SUBCELLULAR LOCALIZATION OF HEXOKINASES IN HUMAN BREAST CANCER AND NONPATHOLOGICAL BREAST TISSUE

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Subcellular distribution of hexokinase (HK) isoenzymes in 22 human breast cancers (21 primary cancers and 1 axillary metastatic growth) and 7 non-pathological human mammary gland tissue samples was studied with starch gel electrophoresis on isolated cell fractions obtained by differential centrifugation. Fractions used were cytosol, mitochondria and microsomes. A comparison of two methods for detecting HK activity was made, yielding different results regarding HK II and HK III. A method based on the ability of NADPH to fluoresce in UV gave a constant pattern of HK isoenzymes. In non-pathological breast tissue, only HK I was seen, i.e. in the cytosol and the microsomal fractions. HK I was also seen in all fractions of the cancers, but another more anodal band of HK I, as well as HK II and HK III, consistently appeared in the cytosol fractions. The more commonly used staining technique with tetrazolium dye revealed HK II in 45 % and HK III in 50 % of the samples. The pattern of HK isoenzymes in the cancers was the same irrespective of estrogen and progesterone cytosolic receptor contents and the histology of the tumors. The fluorescence method is, therefore, much more sensitive than the tetrazolium technique for detecting HK activity after electrophoresis and could explain difference in results obtained by various laboratories.

In human breast cancer the activity of hexokinase (HK, hexose - ATP - phosphotransferase, E.C. 2.7.1.1.) is much higher than in normal breast tissue (Hilf *et al.*, 1969, 1970, 1973; Szutowicz *et al.*, 1979; Balinsky *et al.*, 1981). Similar changes have been found in many mammalian cancers (Pedersen, 1978). Qualitative changes in HK isoenzymes have also been described in mammalian tumors (Criss, 1971) indicating changes in genetic expression upon transformation.

Among the four hexokinases found in mammalian tissues, designated HK I - HK IV, the most striking change associated with malignancy is seen in HK II (Criss, 1971). HK II has been suggested as a marker for human malignancies (Kamel and Schwarzfischer, 1975) and though it is not consistently found in all human malignancies of the same tissue origin, there is a definite trend in that direction (for a review, see Balinsky, 1980). More recent studies support this view (Kraaijenhagen *et al.*, 1980; Beemer *et al.*, 1982; Balinsky *et al.*, 1983); only the last study deals with human breast cancer. However conflicting reports on the subject have also been published (Kikuchi *et al.*, 1972; Marshall *et al.*, 1979). The appearance of HK II in fetal tissue of human origin has also been observed (Rogers *et al.*, 1975; Balinsky, 1980).

In addition to isoenzymic changes of HK in mammalian cancers, a difference in subcellular distribution has also been observed although this has mainly been studied in non-human cancers. Binding of HK to mitochondria is well established in many rapidly growing tumor cells (for a review, see Pedersen, 1978).

Bartley *et al.* (1975) investigated subcellular distribution of hexokinase in mammary adenocarcinomas and normal mammary glands of lactating mice. They observed that HK I was the predominant mitochondrially-bound form in the cancers, whereas HK II accounted for most of the mitochondrial binding in the lactating gland.

In virgin mice, little if any hexokinase activity could be demonstrated in the mitochondrial fraction (Kopelovich *et al.*, 1966; Bartley *et al.*, 1966). Similar results were obtained in rats (Shatton *et al.*, 1965). Hilf *et al.* (1973) investigated hexokinase activity in relation to estrogen receptor content in human breast cancer, but found no correlation.

We investigated the subcellular distribution and HK isoenzyme pattern of human breast cancer and non-pathological breast tissue and compared these parameters to the histology of the cancers and their estrogen and progesterone cytosolic receptor content. A comparison of two different techniques for detecting HK activity was made.

MATERIAL AND METHODS

Samples

Samples of malignant and non-pathological breast tissue were obtained from the Surgical Department of the University Hospital of Iceland. In addition, *post-mortem* samples (non-pathological) were obtained from the Department of Pathology, University Hospital of Iceland.

Malignant tumors were immediately put in ice-cold saline in the operating room. All tumors were histologically inspected for necrosis and fat which were removed. Samples were washed as free of blood as possible with ice-cold saline before and after mincing. Tumors were either quick-frozen in CO₂ and kept at -70° C until use, or, if used fresh, immediately homogenized and centrifuged to yield isolated fractions.

Seven *non-pathological* samples were obtained from breasts removed by radical mastectomy. Fat was excised as completely as possible macroscopically and samples histologically inspected for fat and malignant growth. Samples used contained up to approximately 30 % fat. Non-pathological samples were treated in the same way as tumor samples. No difference in isoenzyme pattern was observed between freshly prepared

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samples and previously frozen ones. Three *post-mortem* samples (non-pathological) were taken at autopsy 6-12 h after death. They were only centrifuged to eliminate cell debris, nuclei and whole cells.

Fractions were lyophilized after centrifugation for storing and concentration. They were kept desiccated at -70°C . No difference in isoenzyme pattern was detected before and after lyophilization.

Preparation of isolated fractions

All work on sample preparation was done at $0-4^{\circ}\text{C}$. The procedure for fractionation of tissue was based on the method used by Drouin (1980). Samples which had been frozen were thawed in isotonic saline in an ice-bath. The tissue was minced and washed in homogenization buffer. Homogenization was done either in buffer I A (0.25 M sucrose, 0.15 M KCl, 10 mM Tris: HCl (pH 7.5), 1 mM EDTA, 0.5 mM DTT (dithiothreitol), or in buffer I B (10 mM Tris: HCl (pH 7.4), 1.5 mM EDTA, 0.5 mM DTT). Both buffers were suitable for use. Homogenization was done with a Polytron[®] homogenizer for 3×8 sec at 1-min intervals to allow cooling of the specimen in an ice-bath. The homogenate was spun at 1,000 g for 10 min to remove cell debris, nuclei and whole cells. The supernatant was spun at 15,000 g for 15 min to yield a mitochondrial pellet and supernatant for cytosol and microsomal isolation. Then the supernatant was spun at 105,000 g for 1 h and supernatant was again taken and is referred to as cytosol. The pellet was gently resuspended in buffer II (0.25 M sucrose, 10 mM Tris: HCl (pH 7.5), 1 mM EDTA, 0.5 mM DTT) and spun again at 105,000 g for 1 h. This was repeated three times and the supernatant in each case was measured for LDH (lactate dehydrogenase) activity, which was used as a marker for cytosol contamination. The final pellet was retained and is referred to as microsomes. The mitochondrial pellet was resuspended gently in buffer II and laid over an equal volume of buffer III (0.44 M sucrose, 10 mM Tris: HCl (pH 7.5), 1 mM EDTA, 0.5 mM DTT) and spun at 3,000 g for 15 min. The pellet was resuspended gently in buffer II and spun at 20,000 g for 10 min. This was repeated twice and the supernatant kept for LDH measurement each time; the final pellet is referred to as the mitochondrial fraction. Both the mitochondrial and microsomal fractions were checked by electron microscopy. LDH was measured at the Department of Clinical Biochemistry, University Hospital of Iceland, by a kinetic method according to the Scandinavian recommendations.

Starch gel electrophoresis

Horizontal starch gel electrophoresis was done in the Tris/EDTA/Mg/Maleate system, pH 7.4, as described by Spencer *et al.* (1964). The electrophoretic run was similar to that described by Povey *et al.* (1975): 20 V/cm for 4 h at 0°C on 10% starch gel (W/V). Samples were sucked into filter paper (Whatman No. 3) 0.7×0.5 cm and embedded in the gel at the origin. Sample concentrations were 25 mg lyophilate/ml in the case of cancers, but 80 mg lyophilate/ml of non-pathological and *post-mortem* samples. Microsomes and mitochondria were treated with 1.5% Triton X-100 v/v, with 0.3 M KCl for 1-2 h, before electrophoresis was carried out. Triton X-100 did not affect isoenzymes of HK as indicated by its lack of effect on the cytosol isoenzyme pattern when

used there as a control. Gels were cut into slices approximately 2.5 mm thick so that the samples could be stained by two different methods for detection of HK activity. One was stained with a solution of similar composition to that described by Povey *et al.* (1975): ATP 45 mg, NADP 7.5 mg, MTT (tetrazolium bromide) 7.5 mg, phenazine methosulfate 5 mg, glucose 6-phosphate dehydrogenase (140 units/ml) 60 μl , made up in a solution of 10 ml of 0.1 M Tris: HCl, pH 7.5, mixed at 45°C with 30 ml of the same buffer and 10 ml 0.02 M MgCl_2 , containing 1% agar. The final concentrations of glucose in solution on respective halves of the gels were 0.001 M and 0.1 M to reveal possible HK IV activity. The gel was incubated in the dark, at 37°C , for up to 16 h.

The other staining method used was based on that described by Kaplan and Beutler (1968), who used the property of NADPH to fluoresce upon illumination with UV. The staining solution without the tetrazolium dye was used, and the gels were transilluminated at intervals of 30 min with UV of 302 nm wavelength after incubation at 37°C from 30 min to 16 h. Gels were also stained with blank solution containing the same materials as the staining solutions except for ATP and/or glucose to detect possible non-HK activity.

Cytosolic receptor assay

Estrogen receptors (ER) and progesterone receptors (PgR) were measured by a dextran-coated charcoal (DCC) method (McGuire *et al.*, 1973), using Insta-Gel as aqueous counting scintillant. ER and PgR concentration of more than 8 femtomoles/mg protein is designated positive. Protein concentration was measured by the Bio-Rad protein dye binding assay, using bovine serum albumin as a standard.

Chemicals

Chemicals were obtained as follows: Starch-hydrolysed (Connaught Laboratories Ltd., Willowdale, Ontario, Canada); DDT (Calbiochem, San Diego, CA, USA); [2,4,6,7, $-^3\text{H}$]estradiol (Amersham International, Amersham, England); net-555-promegestone [17 α -methyl- $-^3\text{H}$] and NLP-004 nonlabelled promegestone (New England Nuclear, Dreieichenhain, FRG); diethylstilbestrol and hydrocortisone (The State Drug Monopoly of Iceland); Insta-Gel (Packard Instruments AB, Bandhagen, Sweden); protein standard bovine gamma globulin and protein dye (Bio-Rad Laboratories, Munich, FRG); and tris (hydroxymethyl) aminomethane (Merck, Darmstadt, FRG). Materials for LDH measurements were obtained from Boehringer, Mannheim, FRG. All other chemicals and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Company, Saint Louis, Mo, USA).

RESULTS

The hexokinase isoenzyme pattern in breast cancer samples was quite different from that of the non-pathological breast tissue (Fig. 1). Using the fluorescence method a constant isoenzyme pattern in the cancer samples was seen: in the cytosols three hexokinases were found, HK I, having two zones of activity, HK II and HK III, a definite reaction always appearing within 1 h. No signs of HK IV were seen, either in freshly prepared samples or in previously frozen ones.

FIGURE 1 – Electrophoretic pattern of hexokinase isoenzymes, in breast cancer (a,b,c), and non-pathological breast tissue (d,e,f), detected by the fluorescence technique in 0.001 M glucose. (a) Microsomal fraction; (b) mitochondrial fraction; (c) cytosol fraction; (d) microsomal fraction; (e) mitochondrial fraction; (f) cytosol fraction. Samples were placed at the origin (O) and the run was towards the anode (+). For details refer to the text.

In the mitochondrial fraction only HK I was found, the more cathodal band. This same band was also the only clearly demonstrable HK isoenzyme in the microsomal fraction. In two cases, a more anodal band of HK II was seen in the cytosol, probably HK II_f (Balinsky *et al.*, (1983).

In the non-pathological breast tissue only HK I (the more cathodal band) was seen, constantly appearing in both the cytosolic and the microsomal fractions (in one sample there were traces of this band in the mitochondrial fraction, seen after prolonged staining of more than 4 h visualized by the fluorescence technique). The non-pathological autopsy material also showed only HK I. In only one non-pathological sample could traces of HK II be seen in the microsomal fraction after prolonged staining, visualized by the fluorescence technique. No sign of it was seen in the cytosolic or the mitochondrial fraction of the same sample, even though electrophoresis was done with 100 mg lyophilate/ml.

The more commonly used tetrazolium dye method always revealed both bands of HK I in the cancer cytosols as well as the more cathodal band in the microsomal and mitochondrial fractions. On the other hand, the appearance of HK II and HK III by that method was not a constant finding, a definite band of HK II only being observed in 10 of 22 samples (45 %) and of HK III in 11 of 22 samples (50 %).

The constant HK isoenzyme pattern was independent of the cytosolic ER and PgR content. The isoenzyme pattern was also the same irrespective of the histology of the cancers.

DISCUSSION

In this study of isoenzyme pattern and subcellular localization of hexokinase in human breast cancer and non-pathological breast tissue we obtained different results with the two staining techniques.

The method based on the ability of NADPH to fluoresce in UV to detect HK activity gave a constant shift in isoenzyme pattern of hexokinase from the non-pathological to the malignant state, whereas the more commonly used tetrazolium dye method for detecting HK activity after electrophoresis did not show this constant isoenzyme shift. This difference is not a result of the electrophoresis itself as we used both staining methods after every electrophoretic run (for details, see "Material and Methods").

This difference concerns HK II and HK III and is highly interesting in the light of a recent report of Balinsky *et al.* (1983), who did not observe HK II in all their (non-fractionated) human breast cancer samples. Using the tetrazolium dye staining technique they found HK II in 13 % of primary cancers and in 29 % of metastases. In this study they did not mention HK III or the more anodal band of HK I, which we always found in our samples. This may be due to different electrophoretic systems. The difference we obtained between the two techniques indicates that the sensitivity of the detection system in the electrophoretic study of hexokinase plays an important role. It could also explain conflicting results between laboratories (Kikuchi *et al.*, 1972, Marshall *et al.*, 1979). This may be relevant to studies where HK II did not consistently appear (see Balinsky, 1980).

The binding of HK I to mitochondria of the human breast cancer is consistent with the observations in murine mammary glands by Bartley *et al.* (1966, 1975).

It has been reported that HK I, HK II and HK III are probably coded for by separate loci (Rogers *et al.*, 1975; Povey *et al.*, 1975). The duplication of HK I has been reported in human uterine carcinomas (Marshall *et al.*, 1979), as well as in human fetal tissue (Rogers *et al.*, 1975; Hammond and Balinsky, 1978). It has been suggested that the duplication of HK I in fetal tissue is a secondary modification of the primary enzyme, similar to that observed for some enzymes in rapidly proliferating cells (Rogers *et al.*, 1975). Rapid cell proliferation is common to both cancer and fetal tissue, and it has been suggested that the fetal stage tends to be reproduced in cancer (Balinsky, 1980).

Bennett *et al.* (1978), working on gliomas, observed HK II in neoplasia of high malignancy and a low grade of differentiation, and suggested that it was a consequence rather than the cause of neoplasia. Our findings of a constant isoenzyme pattern of HK in human breast cancer, independent of the histology, might reflect an activation of genes, characteristic for malignant transformation in the breast. The other possibility is that it merely reflects active cell proliferation.

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