Antioxidant and glutathione-associated enzymes in Wilms' tumour after chemotherapy

Abstract The present study demonstrates the activities of antioxidant and glutathione-associated enzymes and the level of glutathione in Wilms' tumour (nephroblastoma) samples after chemotherapy (mainly actinomycin D and vincristine). We observed higher activity of superoxide dismutase in Wilms' tumour compared to adjacent morphologically unchanged kidney. On the other hand, in this tumour lower activities of catalase and the glutathione-associated enzymes glutathione synthetase, γ-glutamyl transpeptidase, glutathione reductase and total glutathione S-transferases (GST) were found. Using isoelectric focusing we separated different forms of GST in tested tissues and revealed lower activities of the basic enzymes in Wilms' tumour, which may be responsible for the decrease of total GST activity. Moreover, we found the acidic isoenzymes to be the predominant class of GST in nephroblastoma. In Wilms' tumours with unfavourable histology a high activity of these isoenzymes together with a high level of GSH were observed. We suggest that these parameters may participate in the known phenomenon of anticancer drug resistance of tumours with unfavourable histology.

Key words Wilms' tumour · Antioxidants · GST isoenzymes

Abbreviations GSH glutathione · GST GSH S-transferase

Introduction

Antioxidant defence systems protecting against oxidative damage are usually changed during carcinogenesis or in tumours (Oberley and Oberley 1986; Sun 1990). The differences in the activities of direct antioxidant enzymes are usually manifested as lowered catalase and superoxide dismutase activities as compared to corresponding adjacent tumour-free tissue (Tisdale and Mahmoud 1983). The decrease of these activities may be a cause of the excess of oxygen free-radicals and the increase of oxidative damage in tumours (Bartoli et al. 1980). A tendency towards reversal was postulated for the activities of glutathione-linked enzymes such as glutathione peroxidase, glutathione reductase, glutathione S-transferase (GST) and, cooperating with them, glucose-6-phosphate (Glc6P) dehydrogenase (Sun 1990). These activities are usually increased in tumours and may eliminate some of active oxygen species as well as a wide variety of compounds including a number of therapeutics. In particular, GST are the enzymes, that have as their main detoxication function the catalysis of the conjugation of glutathione (GSH) to various electrophilic metabolites. On the basis of the differences in pI values, human GST are divided into three major classes: α (basic), μ (near-neutral) and π (acidic) (Mannervik et al. 1985). GST are present with high activities in many tumours and in particular GST π has recently been considered to participate in drug-resistance mechanisms (Tsuchida and Sato 1992). Several studies have indicated that an overexpression of GSH-based detoxification may protect tumour cells against cytostatic drugs and γ-irradiation (Shan et al. 1990) whereas a lower expression may potentiate the effect of these agents (Ozols et al. 1990).
Wilms' tumour (nephroblastoma), an embryonal malignancy of the kidney, is one of the commonest solid tumours of childhood. In Poland, as well as in many European centres, according to SIOP (International Society of Pediatric Oncology) protocols, pre- and postoperative chemotherapy are used during clinical treatment of this tumour. Nephroblastoma often responds well to chemotherapy, however tumours with unfavourable histological features are associated with a high frequency of relapses and death (Beckwith and Palmer 1978; Zuppan et al. 1988).

In this study, we compared the activities of antioxidant and GSH-related enzymes and the level of GSH in Wilms' tumour after chemotherapy with these values in adjacent kidney. In both tissues we also estimated the isoenzyme pattern of GST by the method of high-performance analytical isoelectric focusing. Moreover, we compared the above-mentioned parameters in Wilms' tumour to establish the possible dependence between the enzyme profile and histology.

**Materials and methods**

**Chemicals**

Epinephrine, glucose 6-phosphate, reduced glutathione, oxidized glutathione, Pharmalyte pH 3-10, Coomassie brilliant blue R-250, ammonium persulphate, acrylamide, N, N'-methylenebisacrylamide, markers for isoelectric focusing (pI range 3.6-9.3), glutathione reductase (bakers' yeast), reduced nicotinamide adenine dinucleotide phosphate, nicotinamide adenine dinucleotide phosphate, o-phthalaldehyde, 1-chloro-2,4-dinitrobenzene, adenosine triphosphate, L- e-aminobutyrate, L-7-glutamyl-4-nitroanilide and glycylglycine were purchased from Sigma Chemical Co. (St. Louis, Mo., USA), N, N, N', N'-Tetramethylidylenediamine was purchased from Fluka AG (Buchs SG, Switzerland).

**Patients**

This research was approved by the Ethical Committee at the National Research Institute of Mother and Child. Parents of patients gave their consent to the inclusion of their children in this study. Wilm's tumour and kidney samples were obtained from 26 patients at the time of surgery. As in the other European centres we also diagnosed and treated Wilms' tumours according to SIOP-9 protocols. In our studies, tumours with favourable histology (FH, a low grade of malignancy) were obtained from 3 patients in clinical stage I, with a mean age of 3.3 _±_ 3.0 years. Tumours with unfavourable histology (UH, a high grade of malignancy) were obtained from 8 patients in clinical stages I (1 patient), II (5 patients) or IV (2 patients), with a mean age of 3.0 _±_ 1.8 years. The majority of Wilms' tumours analysed were of typical histology (TH, an intermedicated grade of malignancy) and were obtained from 15 patients in clinical stages I (6 patients), II (7 patients) or III (2 patients), with a mean age of 2.8 _±_ 1.9 years. All patients had received preoperative treatment with actinomycin D, vincristine and, in some cases, doxorubicin and etoposide also. The number of parameters analysed in each sample was dependent on the amount of tissue available.

Preparation of tissues

Representative pieces of tumour tissues without necrotic areas were taken for assay. The tumour and kidney samples were immediately washed in a cold saline solution and homogenized with four volumes of 0.25 M sucrose. The homogenates were centrifuged at 3000 g for 15 min and then at 27000 g for 30 min. Final supernatants were collected and used for enzymatic assays and isoelectric focusing of GST.

For GSH estimation, tissue samples were prepared separately. They were homogenized in four volumes of ice-cold 6.5% trichloroacetic acid and then centrifuged at 10000 g for 10 min. Supernatants for assay were stored at _—_ 70°C.

**Enzyme assays**

The activity of superoxide dismutase (CuZn) was determined spectrophotometrically by inhibition of epinephrine autoxidation at 320 nm (Misra and Fridovich 1972). One enzymatic unit was the amount of enzyme causing 50% inhibition of the initial rate of autoxidation. The catalase activity was assayed by measuring the rate of degradation of 15 mM hydrogen peroxide at 240 nm (Beers and Sizer 1952). One unit of catalase activity was the amount of enzyme that liberates half of the peroxide oxygen from hydrogen peroxide of any concentration in 100 s at 25°C. The activity of GSH peroxidase was assayed with hydrogen peroxide as the substrate at 340 nm (Paglia and Valentine 1967). The GSH reductase activity was monitored by the utilization of NADPH by the enzyme at 340 nm (Bayomy and Rosalki 1976). In the assay of the G6P dehydrogenase activity, the formation of NADPH was recorded at 340 nm at 25°C (Langdon 1966). The total GST activity and activities of GST isoenzymes were assayed with 1-chloro-2,4-dinitrobenzene as a substrate at 340 nm (Habig et al. 1974). For determination of the GGT activity, L-γ-glutamyl-4-nitroanilide and glycylglycine were used as substrate and acceptor molecule respectively. The absorbance of the product (4-nitroaniline) was measured at 405 nm (Szasz et al. 1976). The GSH synthetase activity was determined with ATP and L-α-aminoxybutyrate according to the method of Sekura and Meister (1977). The activities of GSH-linked enzymes were expressed as U/mg protein. One unit of enzyme produces 1 μmol product/min. Protein concentrations were measured by the method of Lowry et al. (1951)

**Glutathione**

The GSH content was examined according to the fluorimetric method of Hissin and Hill (1976) with o-phthalaldehyde using a Perkin-Elmer LS 50 luminescence spectrometer. The level of GSH was expressed as μmol/g tissue.

**Isoelectric focusing**

Isoelectric focusing was carried out according to LKB instructions (Anonymous 1986) in the electrophoresis chamber (LKB Multiphor, Bromma, Sweden) on 0.5-mm-thick, 5% polyacrylamide gels containing 6% (w/v) Pharmalyte in the pH range 3-10, 0.05% N, N, N', N'-tetrarmethyl-ethylendiamine and ammonium persulphate. Supernatants of 27 000 g homogenates of tumours or kidney tissues were applied (20 μl two- or fivefold diluted) in adjacent lanes of the gel. Marker proteins in pH range 3.6–9.3 from Sigma were used. Focusing was carried out at 8°C for 1.5 h at constant power (15 W) and increasing voltage (500–1800 V). After focusing, the gels were divided into 5-mm-wide segments along the pH gradient. The elution of the enzymes from every gel slice was carried out in 1.0 cm²