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SIMILAR TOXIC EFFECT OF 1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA ON LYMPHOCYTES FROM HUMAN SUBJECTS DIFFERING IN THE EXPRESSION OF GLUTATHIONE TRANSFERASE M1-1

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Abstract—Sixteen healthy donors were investigated for the presence or absence of glutathione transferase (GST) M1-1 in lymphocytes by immunodetection with polyclonal antibodies against human GST M1-1. Nine out of 16 individuals (56%) were categorized as GST M1-1 positive. Phytohaemagglutinin stimulated lymphocytes from GST M1-1 positive and negative donors were treated with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and compared regarding inhibition of [^{3}H]thymidine incorporation as a measure of cytotoxicity. No significant differences in the effect of BCNU were observed between the two groups, indicating that GST M1-1 is not an important resistance factor for BCNU.

Key words: human lymphocytes; BCNU; glutathione transferase M1-1; immunodetection; cytotoxicity; resistance factor

Drug resistance, both intrinsic and acquired, is an important problem in cancer chemotherapy [1]. Several different cellular systems have been implicated in drug resistance. Commonly discussed mechanisms of resistance to DNA-damaging drugs include reduced drug accumulation, increased drug excretion, cellular drug inactivation and increased DNA repair [2–4].

GSH‡ and its associated enzymes, such as GSTs, have been extensively studied. GSH is one of the most abundant peptides in our body. It can react with a number of electrophilic and genotoxic compounds and thereby inhibit malignant transformation [5]. Numerous GSH detoxication reactions are catalyzed by GSTs [6] and the enzymes have also been implied in the resistance of tumor cells to bifunctional alkylating cytostatic drugs. Four different classes of GST isoenzymes have been identified in the cytosol: GST class Alpha, Mu, Pi and Theta [7]. Mammalian GST isoenzymes belonging to the same class are similar regarding amino acid sequences, as well as in their substrate specificities and sensitivities to inhibitors [8, 9].

The predominant GST isoenzyme in tumors is usually the class Pi enzyme GST P1-1 [10–12]. It is expressed in many human tissues but its functional role remains undefined. One of the substrates giving the highest catalytic activity with this enzyme is acrolein (prop-2-enal), a toxic aldehyde that occurs as an environmental pollutant and as a metabolite of cyclophosphamide [13]. Increased GST P1-1 activity has been demonstrated in human tumor cell lines resistant to mitomycin C, melphalan, *cis*-diamminedichloroplatinum [16] and doxorubicin [14–18].

In distinction from the Pi class, the Alpha class of GSTs is known to contain more than one isoenzyme. Liver tissue contains large amounts of GST A1-1, as do kidney, testis and adrenal glands [19]. It has been shown that L-phenylalanine mustard is a substrate for class Alpha GSTs [20], including human GST A1-1 [21], and increased expression of an Alpha class GST was observed in a chlorambucil resistant cell line [22].

Similarly, the Mu class is composed of several isoenzymes. GST M1-1, previously referred to as GST μ, [7] is characterized by its high efficiency in the detoxication of epoxides [9, 23, 24]. It was discovered in the liver [23], but is expressed also in other tissues including lymphocytes [25]. GST M1-1 shows polymorphism and the enzyme is present in only about 60% of the human population [23, 24, 26]. It has been shown that denitrosation of BCNU is catalysed more efficiently by class Mu GSTs from the rat than by class Pi and Alpha GSTs [27]. It was also demonstrated that while overall GST activity was decreased and class Pi GST was down regulated, the cytosolic concentrations of class Mu GSTs were elevated in rat brain tumor cells resistant to BCNU. Pretreatment of these cells with the GST inhibitors ethacrynic acid or triphenyltin chloride enhanced

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‡ Abbreviations: GST, glutathione transferase; GSH, glutathione; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; FCS, fetal calf serum; [^{3}H]thymidine, ([^{3}H]-methyl)-thymidine; PHA, phytohemagglutinin; TCA, trichloroacetic acid.

the cytotoxic effects of BCNU [27]. These results indicate that class Mu GSTs play a role as a resistance factor against BCNU in the rat.

The aim of this study was to investigate whether the Mu class GST M1-1 acts as a resistance factor against BCNU in human cells, as previously indicated for Mu class enzymes in rat cells. Human lymphocytes differing in the expression of GST M1-1 were tested for their sensitivity to BCNU.

MATERIALS AND METHODS

Lymphocytes from heparinized fresh human blood of 16 healthy donors were collected by differential centrifugation in Ficoll-Isopaque [28]. Samples containing 10^6 lymphocytes were resuspended in water and incubated on ice for 10 min. After sonication (3 × 20 sec), 1:10 of 10 × PBS was added and the suspensions were centrifuged in Eppendorf tubes in a microcentrifuge at 16,000 *g* for 20 min. The supernatants were further centrifuged at 105,000 *g* for 60 min. Crude cytosolic fractions of approximately 200 μg protein were applied to nitrocellulose filters for slot blot analysis. Blocking of filters was made with 3% (w/v) BSA for 1 hr. Antibodies [8] to GST M1-1 or rat GST 3-3 were added at a concentration of 15 μg/mL and incubated for 3 hr. The filters were washed three times for 10 min in washing buffer [0.2% (w/v) BSA, 0.1% (w/v) Triton X-100 and 0.02% (w/v) SDS in PBS] and incubated for 3 hr in [^{125}I]Protein A (Amersham International plc, Amersham, U.K.) for detection of immunocomplexes. The filters were then rinsed three times in washing buffer and once in PBS; after drying the immunocomplexes were visualized by autoradiography.

For the cytotoxicity assays, lymphocytes (0.5–1.0 × 10^6 cells per mL) were stimulated for 48 hr with 6% (w/v) PHA (Difco Laboratories, Detroit, MI, U.S.A.) in Eagle's MEM medium with Earl's salts (Flow Laboratories, Rickmansworth, U.K.) supplemented with 10% (v/v) FCS, 125 IU benzylpenicillin and 125 μg streptomycin per mL of medium. The cells were then exposed for 120 min at 37° to different concentrations of BCNU [obtained as a sterile powder in commercial vials from Bristol Laboratories (Syracuse, NY, U.S.A.) dissolved in 40 μL 99.5% ethanol and diluted in Eagle's MEM medium]. After removal of the drug, the cells were resuspended in 1.5 mL medium and 10 μL [^{3}H] thymidine [5 Ci/mmol, 10 μCi/mL, from Amersham International plc (Amersham, U.K.)] was added. The cells were then incubated for 2 hr. The labeling was terminated by placing the cells on ice and removing the medium containing [^{3}H]thymidine. Following this, cells were precipitated with 5% TCA, washed three times in 5% TCA, resuspended in 70% ethanol, and plated onto circular (2.5 cm diameter) glass fiber filters (Type GF/B, Whatman Ltd, U.K.). Radioactivity on the filters was measured by scintillation counting following release of radioactivity with 0.6 mL Solubilizer in 10 mL scintillation vials (Packard Instrument Company, Downers Grove, IL, U.S.A.).

RESULTS

The presence of GST M1-1 in lymphocytes was determined by examining autoradiographs of nitrocellulose filters for the presence of immunocomplexes with antibodies raised against the enzyme. Out of 16 donors, seven were clearly positive and three clearly negative when tested with antibodies against human GST M1-1. In six samples the signals were weak and it was difficult to decide whether they should be scored as negative or positive. In order to clarify the interpretation of the experimental results, the samples were also tested with antibodies raised against rat GST 3-3, which are known to cross-react with human GST M1-1 [8]. The rationale for using the anti-rat antibodies is that the titer was higher than that of the anti-GST M1-1 antibodies. Therefore, the sensitivity is increased. Further, the antibodies to rat GST 3-3 have been found to cross-react only with class Mu GSTs [8]. Anti-GST 3-3 antibodies gave positive signals with all samples that were clearly positive with anti-GST M1-1 antibodies. In the samples giving weak signals, only those (two out of six) that gave signals with both human and rat antibodies were scored as positive for GST M1-1. Thus, nine of the samples were scored positive and seven negative.

PHA-stimulated lymphocytes from the 16 donors were treated with BCNU at concentrations ranging from 10^{-5} M to 10^{-3} M and cytotoxicity was assayed by measuring the inhibition of [^{3}H]thymidine incorporation. As seen in Fig. 1, BCNU inhibited the [^{3}H]thymidine incorporation to the same extent in GST M1-1 positive as in GST M1-1 negative lymphocytes. Even if the analysis was restricted to a comparison between the clearly GST M1-1 positive and the clearly GST M1-1 negative lymphocytes, the two groups of lymphocytes showed the same sensitivity to BCNU. Thus, the results do not provide any evidence for the assumption that BCNU or an active metabolite of the compound is a substrate for GST M1-1. Nor do the data suggest protection against BCNU by reversible or irreversible binding to the enzyme.

DISCUSSION

Human GST M1-1 was discovered in the liver [23] and has later been shown to be responsible for the catalytic activity towards trans-stilbene oxide in peripheral blood mononuclear lymphocytes [25]. The absence of GST M1-1 in smokers has been correlated with an elevated risk to develop lung cancer [29, 30]. The biological significance of GST M1-1 appears related to its high capacity to conjugate toxic epoxides [24] formed by biotransformation of organic compounds such as benzo(a)pyrene [31].

Evidence that class Mu GSTs are involved in drug resistance was obtained from studies of rat glioma cells with acquired resistance to BCNU and increased levels of class Mu GST isoenzymes [27]. Further indications that class Mu GSTs may be involved in drug resistance were obtained from drug potentiation studies involving ethacrynic acid, an inhibitor of GSTs [27, 32]. This compound efficiently inhibits the class Mu GST M1-1 as well as GST isoenzymes

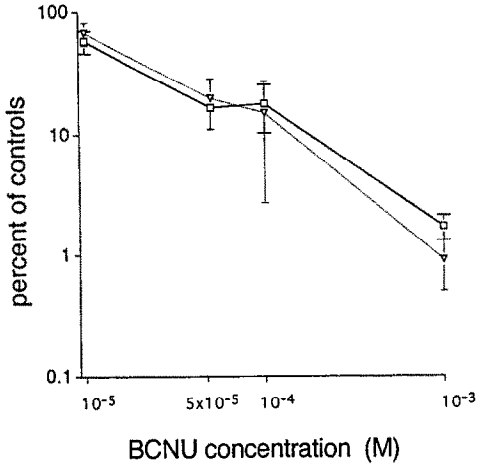

Fig. 1. Cytotoxicity of BCNU as measured by inhibition of [^{3}H]thymidine incorporation in PHA-stimulated lymphocytes. GST M1-1 positive (N = 9) (—□—) and negative (N = 7) (—▽—) individuals. Symbols indicate mean values, bars = SEM.

belonging to other classes, and an inhibitory dose of ethacrynic acid potentiated the effect of melphalan treatment of RPMI 8322 melanoma cells, as demonstrated by increased toxicity as well as increased DNA interstrand cross-linking [32].

In this study we have characterized human lymphocytes from healthy donors with respect to the presence or absence of GST M1-1. The participating individuals could be separated into two different groups, one expressing GST M1-1 (56%) and the other not expressing GST M1-1 (44%). Studies of the human GST class Mu locus has shown a genetic polymorphism in the population such that only 50–60% possess the gene for GST M1-1. Lack of GST M1-1 expression [24] is probably due to homozygosity of a null allele caused by a gene deletion (26, 33–36). However, in characterizing the cellular resistance phenotype determination of the presence or absence of the protein (rather than the gene) is the essential feature.

The inhibition of [^{3}H]thymidine incorporation by BCNU in lymphocytes was similar in GST M1-1 positive and GST M1-1 negative populations of PHA-stimulated lymphocytes, indicating that the human isoenzyme GST M1-1 is not a major resistance factor for BCNU. This finding stands in contrast to the suggested role of the structurally related Mu class GSTs in BCNU-resistant rat glioma cells [27]. However, it is known that even closely related isoenzymes may differ significantly in their functional properties [9]. The absence of detectable GST M1-1 in an individual, does not imply loss of expression of the entire GST class Mu locus, which contains several clustered genes [35–38]. On the other hand, recent data show that another human GST, GST M3-3, may play a role in BCNU resistance [39]. However, it is not known if other class Mu isoenzymes, such as GST M3-3, are expressed in the lymphocytes. It should also be noted that class Mu GSTs may be of importance for resistance to other chemotherapeutic agents, such as nitrogen mustards [40]. Further studies are required to determine the role of GST class Mu as a resistance factor to chemotherapeutic agents.

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