Clin Exp Med (2006) 6:33–37
DOI 10.1007/s10238-006-0091-x

ORIGINAL

A.S. Khayat • L.M. Antunes • A.C. Guimarães • M.O. Bahia • J.A.R. Lemos • I.R. Cabral • P.D.L. Lima M.I.M. Amorim • P.C.S. Cardoso • M.A.C. Smith • R.A. Santos • R.R. Burbano

Cytotoxic and genotoxic monitoring of sickle cell anaemia patients treated with hydroxyurea

Received: 24 June 2005 / Accepted: 4 February 2006

Abstract Very satisfactory results have been obtained with the treatment of sickle cell anaemia with hydroxyurea (HU), an antineoplastic drug. This is because it significantly increases the levels of foetal haemoglobin. Nevertheless, inadequate dosages or prolonged treatment with this pharmaceutical can provoke cytotoxicity or genotoxicity, increasing the risk of neoplasia. We monitored patients under treatment with HU for possible mutagenic effects, through cytogenetic tests (mitotic index and chromosome aberrations) for one year. Checking at two-month intervals, the cytotoxic effect was not evident. There was no evidence of genotoxicity under the conditions of our experiment. However individuals treated with HU should be constantly monitored, as an absence of genotoxicity could be transitory; the mitotic index should also be observed, as an indicator of cytotoxicity.

Key words Hydroxyurea • Sickle cell anaemia • Foetal haemoglobin • Genotoxic monitoring

A.S. Khayat • A.C. Guimarães • M.O. Bahia • J.A.R. Lemos
I.R. Cabral • P.D.L. Lima • P.C.S. Cardoso • R.R. Burbano
Centro de Ciências Biológicas,
Universidade Federal do Pará,
Belém, PA, Brazil

L.M. Antunes
Departamento de Ciências Biológicas,
Faculdade de Medicina do Triângulo Mineiro,
Uberaba, MG, Brazil

J.A.R. Lemos
Centro de Hemoterapia e Hematologia do Estado do Pará,
Belém, PA, Brazil

M.I.M. Amorim
Universidade da Amazônia,
Belém, PA, Brazil

M.A.C. Smith • R.R. Burbano
Disciplina de Genética, Departamento de Morfologia,
Escola Paulista de Medicina,
Universidade Federal de São Paulo,
São Paulo, SP, Brazil

R.A. Santos
Departamento de Genética,
Faculdade de Medicina de Ribeirão Preto,
Universidade de São Paulo, São Paulo, SP, Brazil

R.R. Burbano (✉)
Laboratório de Citogenética Humana,
Departamento de Biologia, Centro de Ciências Biológicas,
Universidade Federal do Pará - Campus Universitário do Guamá,
Av. Augusto Correa 01, CEP 66075-900 Belém, Pará, Brazil
e-mail: rommel@ufpa.br
Tel.: +55-91-88357972
Fax: +55-91-32017601

Introduction

Hydroxyurea (HU) is an antineoplastic and a chemotherapeutic agent widely used for the treatment of myeloproliferative diseases, including myeloid leukaemia, polycythemia vera and essential thrombocytopenia [1, 2]. This drug interrupts the normal mechanism of reduction of ribonucleotides and deoxyribonucleotides through the inactivation of ribonucleotide reductase, limiting DNA biosynthesis [3, 4].

Currently HU is being used as an inductor of foetal haemoglobin (HbF) synthesis, giving good results in the treatment of sickle cell anaemia (SCA) [5, 6] because: (1) it is less toxic than other chemotherapeutic drugs, (2) myelosuppression, the principal side effect of HU, is reversible when treatment is suspended and (3) there is a considerable increase in the production of HbF, generally greater than with most of the known HbF inductors [7].

Though HU has a beneficial effect in the treatment of various pathologies, this drug can also provoke DNA alter-

ations (genotoxic activity), inducing gene and chromosome mutations [8]. Nevertheless, only one study has reported that therapy with HU has a low mutagenic and carcinogenic potential [9]. In this study, HPRT and VDJ assays were used to quantitate acquired somatic DNA mutations in peripheral blood mononuclear human cells after *in vivo* exposure.

We did a cytogenetic study of the genotoxicity and cytotoxicity of HU used to treat SCA patients, determining the frequency of chromosome aberrations and the mitotic index (MI) as criteria for evaluation. This approach is original in the literature.

Material and methods

Eight patients, of both sexes, 7–20 years old, were included in this study. Peripheral blood lymphocytes were collected by venipuncture and these were cultivated for 72 h in complete culture medium containing 76.8% HAM-F10 medium, supplemented with 0.01 mg/ml streptomycin, 0.005 mg/ml penicillin, 19.2% foetal bovine serum and 4% phytohaemagglutinin. The cytological preparations for the analysis of peripheral human blood lymphocyte metaphases were obtained using an adaptation of the technique of Moorhead et al. [10]. The slides were analysed under an optical microscope, to assay for numerical or structural chromosome alterations (gaps, breaks, complex rearrangements and other alterations) [11]. The protocol was approved by the Medical Ethics Committee of the Hemotherapy and Hematology Center of the State of Pará and the patients consented to the use of their blood samples in this work.

Temporary lymphocyte cultures, from the same patients before treatment with HU, were used as controls. After collecting the control blood sample, each patient received a daily dose of HU, determined according to body weight (25 mg/kg/day). These doses were monitored six times (control+six experiments), at two month intervals, totalling 1 year.

The criteria for analysis were: the frequency of chromosome aberrations per 100 metaphases/culture, and the MI, determined by counting the number of metaphases in 2000 lymphoblasts/culture.

Student's *t*-test and the *F* test (ANOVA) were used to make a comparative analysis of the frequencies of chromosome aberrations and to detect variations in the MI, respectively, before and after treatment with HU. The alpha level for significance was established as 5% [12].

Results

We found no *in vivo* cytotoxicity in most of the HU doses. There was a significant reduction in the MI only in the 2nd culture in some patients (the 1st culture was the control, without HU), when the patients had their first contact with the drug, subsequently returning to normal levels. Overall, the MI did not vary significantly ($P>0.05$). The frequency of chromosome aberrations after treatment was not significantly different ($P>0.05$) from the controls (Table 1).

Table 1 Mitotic index (%), frequency and distribution of chromosomal aberrations, number of polyploid cells and number of cells with endoreduplication cells in lymphocyte cultures from patients with sickle cell anaemia. The first culture is the control (before treatment) and the others were performed in intervals of two months during the treatment. 100 cells/patient/culture were analysed for chromosomal aberration and 2000 cells/patient/culture were analysed for MI

Patient	Culture	MI, %	Chromosomal aberration					CA/100 cells	% of CA	PC	NEC
			G'	G''	B'	OA	Total				
I	1st	1.95	2	0	0	0	2	2.0	1.98	1	0
	2nd	0.75	2	0	0	0	2	2.0	1.98	0	0
	3rd	3.00	1	0	0	0	1	1.0	0.99	0	0
	4th	0.55	2	0	1	0	3	3.0	2.97	0	0
	5th	0.90	1	0	0	0	1	1.0	0.99	0	0
	6th	1.20	1	0	0	0	1	1.0	0.99	0	0
	7th	1.50	2	0	0	0	2	2.0	1.98	1	0
II	1st	1.65	5	0	0	0	5	5.0	4.95	0	0
	2nd	2.25	1	0	0	0	1	1.0	0.99	0	0
	3rd	1.80	0	0	0	0	0	0.0	0.00	0	0
	4th	2.05	0	0	0	0	0	0.0	0.00	0	0
	5th	0.60	2	0	1	0	3	3.0	2.97	0	0
	6th	2.25	1	0	0	0	1	1.0	0.99	0	0
	7th	3.60	0	0	0	0	0	0.0	0.00	0	0
III	1st	1.90	2	0	3	0	5	5.0	4.95	0	0
	2nd	1.20	0	0	0	0	0	0.0	0.00	0	0
	3rd	1.95	1	0	0	0	1	1.0	0.99	0	0
	4th	4.50	1	0	0	0	1	1.0	0.99	0	0

Cont. →

Cont. table 1

Patient	Culture	MI, %	Chromosomal aberration					CA/100 cells	% of CA	PC	NEC
			G'	G''	B'	OA	Total				
	5th	0.45	0	0	0	0	0	0.0	0.00	0	0
	6th	1.80	0	0	0	0	0	0.0	0.00	0	0
	7th	3.90	1	0	0	0	1	1.0	0.99	0	0
IV	1st	0.55	2	0	2	0	4	4.0	3.96	0	0
	2nd	1.95	2	0	2	0	4	4.0	3.96	0	0
	3rd	1.95	1	0	2	0	3	3.0	2.97	0	0
	4th	3.60	2	0	4	0	6	6.0	5.94	0	0
	5th	0.75	1	0	4	0	5	5.0	4.95	0	0
	6th	4.50	3	0	2	0	5	5.0	4.95	0	0
	7th	7.20	4	0	2	0	6	6.0	5.94	0	0
V	1st	1.60	1	0	0	0	1	1.0	0.99	0	0
	2nd	0.00	0	0	0	0	0	0.0	0.00	0	0
	3rd	2.85	0	0	0	0	0	0.0	0.00	0	0
	4th	2.25	1	0	0	0	1	1.0	0.99	0	0
	5th	3.00	0	0	0	0	0	0.0	0.00	0	0
	6th	3.60	1	0	0	0	1	1.0	0.99	0	0
	7th	2.55	2	0	0	0	2	2.0	1.98	0	0
VI	1st	0.55	0	0	0	0	0	0.0	0.00	0	0
	2nd	0.60	0	0	0	0	0	0.0	0.00	0	0
	3rd	1.65	1	0	0	0	1	1.0	0.99	0	0
	4th	6.90	2	0	0	0	2	2.0	1.98	0	0
	5th	3.60	0	0	1	0	1	1.0	0.99	0	0
	6th	2.55	2	0	0	0	2	2.0	1.98	0	0
	7th	0.90	2	0	1	0	3	3.0	2.97	0	0
VII	1st	0.45	0	0	0	0	0	0.0	0.00	0	0
	2nd	0.90	1	0	0	0	1	1.0	0.00	0	0
	3rd	3.45	2	0	0	0	2	2.0	0.00	0	0
	4th	2.25	1	0	2	0	3	3.0	2.97	0	0
	5th	1.20	1	0	1	0	1	1.0	0.99	1	2
	6th	2.85	0	0	1	0	1	1.0	0.99	0	0
	7th	2.55	0	0	1	0	1	1.0	0.99	2	0
VIII	1st	1.60	2	1	2	0	5	5.0	4.95	0	0
	2nd	0.45	2	0	1	0	3	3.0	2.97	0	0
	3rd	0.75	1	0	1	0	2	2.0	1.98	0	0
	4th	2.85	2	0	1	0	3	3.0	2.97	0	0
	5th	2.10	0	0	3	0	3	3.0	2.97	0	3
	6th	7.05	0	0	1	0	1	1.0	0.99	0	1
	7th	0.60	0	0	0	1^{tr}	1	1.0	0.99	1	1

CA, chromosomal aberration; *G'*, chromatid gap; *G''*, chromosome gap; *B'*, chromatid break; *OA*, other aberrations; *PC*, polyploid cells; *NEC*, number of cells with endoreduplication

Discussion

Based on clinical evidence, some antineoplastic agents induce tumours when used at therapeutic doses. The most frequent secondary malignancy induced by these agents is acute non-lymphocytic leukaemia, while the incidence of solid tumours is not fully known [13]. For this reason, it is important to monitor the genotoxic effects of antitumoral drugs when they are routinely administered to patients.

Currently, the chemotherapeutic drug HU is being used to treat some haemoglobinopathies, such as SCA, however little is known about its genotoxic effects in these treatments [5, 6]. It is known, however, that HU is mutagenic in rodent cell cultures [14–17].

According to the literature, the clinical symptoms of SCA patients are improved with HU treatment [18, 19], however this treatment can cause teratogenesis, mutagenesis and chromosome aberrations, depending on the doses given. Chromosome alterations have been found in poly-

cythaemia vera (and allied diseases) patients treated with HU [20]. Additionally, the clastogenic potential of HU has been demonstrated *in vitro* in Erlich's tumour ascite cells, which had a high rate of chromosome breaks when cultured with HU [21].

The normal dose of HU given to patients with SCA is about 10–40 mg/kg/day. We used 25 mg/kg/day, and found no toxicity in the cytogenetic assays.

Among the eight SCA patients treated with HU, patient IV (Table 1) had the largest number of chromosome aberrations, though this was not significant as the control culture also had a high rate of chromosome aberrations. This is highly important, as chromosome alterations in the peripheral blood of untreated individuals indicate endogenous chromosome instability. This factor should be taken into account before the patients initiate therapy, as individuals with chromosome instability are predisposed to carcinogenesis.

There was a decrease in MI at the first post-treatment cytogenetic analysis in four patients (I, III, V and VIII). This cytotoxicity is probably due to the action of HU on ribonucleotide reductase, a phenomenon that was not repeated in the subsequent analyses. A physiological stabilisation was observed; it was maintained until the end of the experiment. Apparently the organism adapted to the drug, and after a year of therapy the gradual increase in dose was not enough to inhibit the activity of ribonucleotide reductase.

A lack of genotoxicity in lymphocytes was also found in *in vitro* studies carried out by our group after HU treatment of healthy subjects and SCA patients [22, 23]. These results support the hypothesis that HU has no direct effect on DNA.

It is known that mammalian cells acquire resistance to many cytotoxic drugs through amplification of the target protein genes [24]. As the absence of cytotoxicity in the subsequent administration first dose was observed in all the patients (3rd culture in Table 1), a mutation at the ribonucleotide reductase gene is unlikely, because it would have to have occurred in all patients. However, this phenomenon needs to be evaluated, because resistance to HU could be induced by a step by step dose increase [25].

This study was carried out on eight patients studied over a limited period of time. In SCA patients, medication with HU is expected to be maintained over several years, thus it is clear that cytogenetic monitoring of these patients should not be interrupted, as a lack of genotoxicity can be temporary. It is also essential to observe the MI; a strong reduction should be reported, so that it can be carefully checked against the haematological indices. Therefore, future studies should be carried out on a larger patient cohort and over a longer time period.

Acknowledgements The authors are grateful to Glorita Santos, Olga and Bruna Khayat for supporting this work. Thanks to Centro de Hemoterapia e Hematologia do Estado do Pará, which kindly provided the samples. The first author was supported by a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior scholarship and by the Universidade Federal do Pará. His work was supported by Financiadora de Estudos e Projetos (FINEP/CT-INFRA) grants 0927-03.

References

1. Elliott MA, Tefferi A (2005) Thrombosis and haemorrhage in polycythaemia vera and essential thrombocythaemia. Br J Haematol 128:275–290
2. De Vita VT, Hellman S, Rosenberg SA (2001) Cancer: principles and practice of oncology, 6th edn. Lippincot Raven Publishers, Philadelphia
3. Graslund A, Ehrenberg A, Thelander LJ (1982) Characterization of the free radical of mammalian ribonucleotide reductase. Biol Chem 257:5711–5715
4. Lori F, Lisziewicz J (2000) Rationale for the use of hydroxyurea as an anti-human immunodeficiency virus drug. Clin Infect Dis 30:193–197
5. de Montalembert M (1999) Hydroxyurea and other agents stimulating synthesis of fetal hemoglobin. Pathol Biol 47:55–58
6. Hoppe C, Vichinsky E, Quirolo K, Van Warmerdam J, Allen K, Styles L (2000) Use of hydroxyurea in children ages 2 to 5 years with sickle cell disease. J Pediatr Hematol Oncol 22:330–334
7. Smith RD, Li J, Noguchi CT, Schechter AN (2000) Quantitative PCR analysis of HbF inducers in primary human adult erythroid cells. Blood 95:863–869
8. Donehower RC (1992) An overview of the clinical experience with hydroxyurea. Semin Oncol 19:11–19
9. Hanft VN, Fruchtman SR, Pickens CV, Rosse WF, Howard TA, Ware RE (2000) Acquired DNA mutations associated with in vivo hydroxyurea exposure. Blood 95:3589–3593
10. Moorhead PS, Nowell PC, Mellmam WJ, Battips DM, Hungerford DA (1960) Chromosome preparations of leukocytes cultured from human peripheral blood. Exp Cell Res 20:613–616
11. Rabello-Gay MN, Rodrigues MAR, Monteleone-Neto R (1991) Mutagênese, teratogênese e carcinogênese: Critérios de avaliação. Sociedade Brasileira de Genética, Ribeirão Preto
12. Ayres M, Ayres Jr M, Ayres DL, Santos AS (2000) Bioestat 2.0: Aplicações Estatísticas nas Áreas das Ciências Biológicas e Médicas, 2nd edn. Sociedade Civil Mamirauá-CNPq, Belém
13. Evans RM (1985) Guidelines for handling parenteral antineoplastics. JAMA 253:1590–1592
14. Ziegler-Skylakakis K, Schwarz LR, Andrae U (1985) Microsome- and hepatocyte-mediated mutagenicity of hydroxyurea and related aliphatic hydroxamic acids in V79 Chinese hamster cells. Mutat Res 152:225–231
15. Amacher DE, Turner GN (1987) The mutagenicity of 5-azacytidine and other inhibitors of replicative DNA synthesis in L5178Y mouse lymphoma cell. Mutat Res 176:123–131
16. Wangenheim J, Bolcsfoldi G (1988) Mouse lymphoma L5178Y thymidine kinase locus assay of 50 compounds. Mutagenesis 3:193–205

17. Matsuoka A, Lundin C, Johansson F, Sahlin M, Fukuhara K, Sjoberg BM, Jenssen D, Onfelt A (2004) Correlation of sister chromatid exchange formation through homologous recombination with ribonucleotide reductase inhibition. Mutat Res 547:101–107
18. Charache S (1991) Hydroxyurea as treatment for sickle cell anemia. Hematol Oncol Clin North Am 5:571–583
19. de Montalembert M, Belloy M, Bernaudin F, Gouraud F, Capdeville R, Mardini R, Philippe N, Jais JP, Bardakdjian J, Ducrocq R, Maier-Redelsperger M, Elion J, Labie D, Girot R (1997) Three-year follow-up of hydroxyurea treatment in severely ill children with sickle cell disease. The French Study Group on Sickle Cell Disease. J Pediatr Hematol Oncol 19:313–318
20. Weinfeld A, Swolin B, Westin J (1994) Acute leukaemia after hydroxyurea therapy in polycythaemia vera and allied disorders: prospective study of efficacy and leukaemogenicity with therapeutic implications. Eur J Haematol 52:134–139
21. Li JC, Kaminskas E (1987) Progressive formation of DNA lesions in cultured Ehrlich ascites tumor cells treated with hydroxyurea. Cancer Res 47:2755–2758
22. Lima PDL, Cardoso PCS, Khayat AS, Bahia MO, Burbano RR (2003) Evaluation of the mutagenic activity of hydroxyurea on the G1-S-G2 phases of the cell cycle: an in vitro study. Genet Mol Res 2:328–333
23. Khayat AS, Guimarães AC, Cardoso PCS, Lima PDL, Bahia MO, Antunes LMG, Burbano RR (2004) Mutagenicity of hydroxyurea in lymphocytes from patients with sickle cell disease. Genet Mol Biol 27:115–117
24. Cocking JM, Tonin PN, Stokoe NM, Wensing EJ, Lewis WH, Srinivasan PR (1987) Gene for m1 subunit of ribonucleotide reductase is amplified in hydroxyurea-resistant hamster cells. Somat Cell Mol Genet 13:221–233
25. Choy BK, McClarty GA, Chan AK, Thelander L, Wright JA (1988) Molecular mechanisms of drug resistance involving ribonucleotide reductase: hydroxyurea resistance in a series of clonally related mouse cell lines selected in the presence of increasing drug concentrations. Cancer Res 48:2029–2035