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GENOTOXIC EFFECTS OF IRINOTECAN AND CISPLATIN ON THE HEALTHY CELLS OF MICE EVALUATED BY ALKALINE COMET ASSAY

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Summary

The purpose of cytostatic agents is to act exclusively upon tumor cells, and to inhibit growth or induce tumor cell death by impairing their cell cycle progression. However, the majority of these agents are not specific in their action, and subsequently produce toxic effects on healthy tissues causing significant adverse events in both patients and health professionals exposed to these drugs. Various cytogenetic and molecular biology assays play an important role in the assessment of genotoxic effects related to antineoplastic drugs. Within a short period after exposure to a potentially genotoxic agent, these assays are able to assess the level of cellular DNA damage and/or to monitor the dynamics of DNA repair. Sensitive techniques, such as alkaline comet assay, are of special importance in the detection of primary DNA damage occurring in individual cells regardless of the cell cycle phase. The aim of the study was to assess and compare DNA damage that irinotecan and cisplatin induce in peripheral leukocytes, and normal kidney, liver and brain cells of Swiss albino mice. The results show that both cytostatics produce statistically significant DNA damage in normal cells compared to the control group. Compared to irinotecan, cisplatin has a significantly more potent genotoxic effect on these cells, which may be attributed to various mechanisms of action of the studied drugs.

KEY WORDS: *genotoxicity, irinotecan, cisplatin, mice, alkaline comet assay*

PROCJENA GENOTOKSIČNIH UČINAKA IRINOTEKANA I CISPLATINA NA ZDRAVE MIŠJE STANICE PRIMJENOM ALKALNOG KOMET TESTA

Sažetak

Po svojoj namjeni citostatici bi trebali djelovati isključivo na tumorske stanice, te narušavanjem njihovog staničnog ciklusa spriječiti rast ili izazvati smrt tih stanica. Međutim, većina ovih lijekova je u svom djelovanju nespecifična, zbog čega se toksične posljedice odražavaju i na stanicama zdravih tkiva, a rezultat toga su značajne nuspojave u bolesnika i osoba koje su profesionalno izložene tim lijekovima. U procjeni genotoksičnih učinaka antineoplastičnih lijekova značajnu ulogu imaju različiti citogenetični i molekularno-biološki testovi. Pomoću njih u kratkom vremenskom razdoblju nakon izlaganja nekom potencijalno genotoksičnom agensu, možemo procijeniti razinu oštećenja stanične DNA i/ili pratiti dinamiku njenog popravka. Posebnu važnost imaju tehnike poput alkalnog komet testa koje omogućavaju osjetljivo otkrivanje primarnih oštećenja DNA u pojedinačnim stanicama, neovisno o fazi staničnog ciklusa. Cilj našeg istraživanja je bio ustanoviti i usporediti oštećenja DNA koja izazivaju irinotekan i cisplatina na leukocitima periferne krvi, na zdravim stanicama bubrega, jetre i mozga Swiss albino miševa. Sukladno rezultatima istraživanja oba citostatika dovode do

statistički značajnih oštećenja DNA spomenutih zdravih stanica u odnosu na kontrolnu skupinu. Međusobno uspoređujući irinotekan i cisplatinu možemo zamijetiti da cisplatina ima statistički značajno jači genotoksični učinak od irinotekana na spomenute stanice, što pripisujemo različitim mehanizmima djelovanja promatranih citostatika.

KLJUČNE RIJEČI: *genotoksičnost, irinotekan, cisplatin, miševi, alkalni komet test*

INTRODUCTION

The administration of numerous antineoplastic drugs represents one of the fundamental treatment modalities for malignant tumor diseases. Referring to their purpose, these drugs should exclusively act upon tumor cells, and by impairing their cell cycle progression, inhibit growth or induce tumor cell death in their phase of active growth. Studies to date, however, show that the majority of these drugs are very non-specific in their action, and therefore after their administration, their toxic effects on healthy tissues cause significant adverse events in both patients and health professionals exposed to these drugs. The modern therapy of malignant tumor diseases uses over fifty kinds of cytotoxic drugs, with a wide variety of chemical substances. Some of commonly used cytotoxic drugs, unfortunately, have a low therapy index and a high potency of inducing adverse side effects. Good knowledge of pharmacology of cytostatic treatments, their interaction with other drugs and clinical pharmacokinetics is an indispensable tool for their safe and effective use. The administration of any chemotherapy drugs is accompanied with an adequate effect on both tumor and normal cells. The ratio between tumor cell and normal cell responses to a certain dose is called a therapy index. A reduced therapy index limits the usability of many chemotherapy drugs. Toxicity to healthy tissue that prevents an increase in dose is called a dose-limiting toxicity. Compared to normal, cytostatics have superior effect on malignant cells as these cells divide and synthetisize DNA faster. On the other hand, normal cells compared to malignant ones recover much more rapidly for their preserved cell repair mechanisms.

Cytostatics are metabolized mostly through the cytochrome P-450 enzyme pathway in the liver and some other tissues. In case bioactivation does not occur in the liver, the entry of cytostatics into the cell should not necessarily lead to cytotoxic activity. Metabolites are excreted via urine, and concurrent administration of other nephrotoxic drugs can enhance cytostatic toxicity and result in mielosuppression, effects on CNS and alike.

Irinotecan is a semisynthetic derivative of camptothecin, and an inhibitor of DNA topoisomerase I. The DNA topoisomerases are nuclear enzymes that reduce torsional stress in supercoiled DNA, allowing selected regions of DNA to become sufficiently untangled and relaxed to permit DNA replication, recombination, repair, and transcription. Topoisomerase I binds covalently to double-stranded DNA through a reversible trans-esterification reaction. By blocking topoisomerase I, irinotecan produces irreversible DNA damage and inhibit tumor cells from dividing. At least 43% of irinotecan bind to plasma proteins, and convert into SN-38 via liver carboxylesterases. Biliary excretion is a major elimination pathway for irinotecan (1).

Cisplatin enters the cell by diffusion. Its platin complex reacts with DNA causing cross-linking between DNA strands or within the strand itself. Guanine sites are shown to be very reactive, which results in the formation of cross-links between neighboring guanines or guanine-adenine from the same strand of DNA. The formation of cross-links between two DNA strands is a slower process that occurs less frequently. Adducts inhibit both DNA replication and DNA transcription, causing its breakage and coding errors. They also evoke helix disorders that result in DNA repair inhibition. The enzymatic recovery of bridges developed through the action of cisplatin includes the excision of the affected base, insertion of a new base and the reformation of the damaged base by means of enzyme activity (2-4).

In the assessment of genotoxic effects related to antineoplastic drugs, various cytogenetic and molecular biology assays play a significant role. Within a short period after exposure to a potentially genotoxic agent, these assays are able to detect and assess the level of cellular DNA dam-

age and/or to monitor the dynamics of DNA repair. Techniques, such as alkaline comet assay, play a special role as they enable sensitive detection of primary DNA damage in individual cells, independent of the cell cycle phase (5).

Method. The study was conducted at the Department of Animal Physiology, Faculty of Science, University of Zagreb, Croatia in 2005. The study included healthy mice.

Mice. Animal studies were carried out according to the Relevant Croatian guidelines (Law on the Welfare of Animals, OG # 19, 1999) and in compliance with the Guide for the Care and Use of Laboratory Animals, DHHS Publ. # (NIH) 86-123. Male albino mice of the Swiss strain, weighing 20-25 grams, from our conventional mouse colony were used. In all experiments, mice were of the same sex and were approximately 2 months old at the beginning of each study. The animals were kept not more then five to a cage and were maintained on a pellet diet and water *ad libitum*.

Campto (Aventis Pharma LTD, UK)

Cisplatin (Cisplatin, Pliva, Zagreb, Croatia).

Experimental design. The mice were divided into three groups. Each group comprised of six mice. Healthy mice were used as a control. The second group of mice was injected with cisplatin 5 mg/kg and the third group received irinotecan 50 mg/kg intraperitoneally for three consecutive days.

On Day 3, the animals were sacrificed by cervical dislocation, and the tissues and blood were taken for analysis.

For the comet assay, peripheral blood, brain, liver, and kidney were taken from all mice for analysis. Blood samples were collected using a micropipette, after the tail vein was cut. The liver, kidney and brain tissues were pressed through the screen in the homogenization buffer pH 7.5 [0.075 M NaCl (Kemika) and 0.024 M Na_2EDTA (Sigma)], and cooled to 4°C; the used ratio was 1 gr tissue to 1 ml buffer.

The comet assay was carried out under alkaline conditions; following a modification by Singh et al (6). Fully frosted slides (Surgipath, USA) were covered with 1% normal melting point (NMP) agarose. After solidification, the gel was scraped off from the slide. The slides were then coated with 300 µl 0.6% NMP agarose. When this layer had solidified, a second layer containing the whole blood samples, the brain cells, liver cells or renal cells mixed with 100 µl 0.5 % low melting point (LMP) agarose was placed on slides. After 10 minutes of solidification on ice, the slides were covered with 0.5 % LMP agarose. The slides were then processed as follows: 1. immersed for 1 h in ice-cold freshly prepared lysis solution [1% Na sarcosinate (Sigma), 2.5 M NaCl (Kemika), 100 mM Na_2EDTA (Sigma), 10 mM Tris-Hcl (Sigma), 1% Triton X-100 (Sigma) and 10% dimetyl sulfoxide (Sigma)] pH 10] added to lyses cells and allow DNA unfolding; 2. placed on a horizontal gel-electrophoresis tank (Life Technologies Ltd), facing the anode. The unit was filled with fresh electrophoretic buffer [0.3 M NaOH (Kemika), 1 mM Na_2EDTA (Sigma) pH 13.0] and the slides were set in this alkaline buffer for 20 min to allow DNA unwinding and expression of alkali-labile sites. Denaturation and electrophoresis were performed at +4°C under dim light. Electrophoresis was carried out for 20 minutes at 25 V and 300 mA. After electrophoresis, the slides were rinsed gently three times with a neutralization buffer (0.4 M Tris-HCl, pH 7.5) to remove excess alkali and detergents. Each slide was stained with ethidium bromide (20 µg /ml) and covered with a coverslip then stored at +4°C in sealed boxes until analysis.

Images of 100 randomly selected cells (fifty counts on each duplicate slide) were analyzed for each sample, a total of 600 cells of the same kind from every group. Cells were examined at × 160 magnifications in a fluorescence microscope (Opton, West Germany). To quantify the DNA damage, the tail length was evaluated (length of DNA migration). Tail length is related directly to the DNA fragment size. Comets were visually inspected and then each comet attached a value of 0-4 according to the degree of damage. Although these units are arbitrary, they can be related to the relative tail intensity which itself is a function of break frequency.

The cells without DNA lesion were classified as category 0 and the ones with maximum tail length and DNA lesions as category 4 (7).

The present study is aimed at determining *in vivo* a possible genotoxic effect of irinotecan and cisplatin on peripheral blood leukocytes

(PBL), kidney, liver and brain cells of healthy Swiss albino mice.

Statistical analysis. The results from the comet assay were analyzed using the descriptive statistical methods, as shown in Figure 1 below. Chi-square test was used for statistical analysis of differences between particular groups.

RESULTS

The results obtained by alkaline comet assay show that both irinotecan and cisplatin have a potent genotoxic effect on healthy cells of the studied mice. Both cytostatics produce a statistically significant damage to DNA in leukocytes, liver cells, kidney cells and brain cells of Swiss albino mice compared to the control group ($p<0.01$) not receiving the treatment. The genotoxic effect of irinotecan compared to cisplatin shows a statistically significant difference ($p<0.01$) in favor of cisplatin which has a more powerful effect on all of the studied cell types. The results are graphically represented in Figure 1.

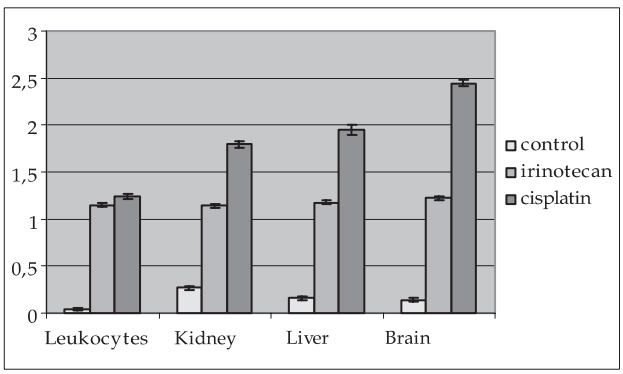

Figure 1. Comparison of comet tail length of healthy cells of Swiss albino mice in vivo exposed to irinotecan or cisplatin as per groups (means±standard error)

DISCUSSION

Irinotecan and cisplatin are powerful cytostatics, and both in the routine use in the treatment of cancer patients. Their genotoxic effect has been known and confirmed in a number of studies, and represents the basic mechanism of antitumor activity (8). However, the mechanism of irinotecan-induced DNA damage in tumor cells is completely different from that of cisplatin.

Irinotecan hydrochloride (CPT-11), which inhibits mammalian DNA topoisomerase I, is an antitumor agent possessing a wide antitumor spectrum, including small cell lung cancer, non-small cell lung cancer, colon cancer, and malignant lymphoma. Following administration, CPT-11 is hydrolyzed to its active metabolite SN-38, predominantly by hepatic carboxylesterase. SN-38 appears to have antitumor activities at least 1000 times as potent as CPT-11 against tumor cells *in vitro*. The complex metabolism of CPT-11 and SN-38 has an impact on the interindividual variability in the pharmacokinetics and toxicity. In the liver, a proportion of SN-38 is subsequently conjugated to SN-38 glucuronide by UDP-glucuronyltransferase. Furthermore, some of the SN-38G are deconjugated to SN-38 by enterobacterial b-glucuronidase in the colon (9).

Cisplatin is not a proper representative of alkalizing chemotherapy drugs, but it is frequently assigned to that group because of its way of action. Its genotoxic effects are evident as cisplatin is an efficient producer of interstrand, intrastrand and monofunctional adduct cross-linking in DNA, consequently increasing the apoptotic cell count. Although alkylations of DNA could occur at any cycle stage, cytoxicity is highest in cells progressing through the cell cycle, such as the tumor cells, affecting the healthy cells as well because of insufficient selectivity.

Since chemotherapy drugs are not selective to tumor cells, the patient's healthy cells are also damaged. Research studies conducted on humans do not give a realistic illustration of the effect of chemotherapy drugs, since the patients differ by age, gender, living conditions, habits and exposure to stress factors and medication administration. The study of the genotoxic effect of irinotecan and cisplatin was conducted on inbred Swiss albino mice under strictly controlled conditions. They were all of the same age, gender, living conditions, nutrition, constant temperature and our results gave a complete picture of *in vivo* genotoxic effects of irinotecan and cisplatin on the leukocytes, kidney cells, liver cells, and the brain cells of healthy mice.

The results of our study show that the genotoxic effect of cisplatin on the healthy cells of Swiss albino mice is significantly more potent compared to irinotecan. In fact, the mechanism of

cisplatin action leading to alkylation and direct DNA damage in both tumor and healthy cells might be responsible for such severe damage. Irinotecan is a newer generation cytotoxic agent, which besides its powerful effect on tumor cells, produces damage in healthy cells as well, however, to a much lesser extent. This study raises new questions about toxicity cytostatics produce to normal cells of various tissues, and the possibility of their DNA repair, as it has been known that the healthy cell repair mechanisms are much better preserved. An open question regarding the possibility of developing secondary neoplasms several years after the therapy completion, however, remains, for the complete repair of DNA in all cells cannot often be achieved.

CONCLUSION

The alkaline comet assay has shown to be a sensitive, simple and fast visual method to detect DNA damage in leukocytes, kidney cells, liver cells and brain cells after irinotecan and cisplatin therapy administered to healthy Swiss albino mice. Although this is only a preliminary study, there is no doubt that it makes a contribution to a better knowledge of cytostatic agents for routine use, and raises questions that require answers in the future.

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