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Polyamines as biomarkers of the antitumoral activity of Bursera fagaroides
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POLYAMINES AS BIOMARKERS OF THE ANTITUMORAL ACTIVITY OF Bursera fagaroides

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SUMMARY

In normal and tumoral cells, the polyamines (PAs) putrescine (Pu), spermidine (Spd) and spermine (Spm) are required in multiple fundamental cell cycle functions. High levels of PAs have been reported in many types of cancer, which is why they were proposed as biomarkers of cancer growth. In the present work, their utility as biomarkers of the evolution of the murine L5178Y lymphoma is reported in different body fluids, cells and tissues. Findings were also applied to the follow-up of the previously reported anti-tumor effect of Bursera fagaroides. Cation exchange chromatography was used to determine the PAs levels in urine, peritoneal cells, circulating lymphocytes, spleenocytes, mesotheliomas and liver of BALB/c mice at days 10, 17 and 24 of tumoral evolution. PAs levels were also measured in urine from mice treated, intraperitoneally or orally, with the hydroalcoholic extract of the bark of B. fagaroides. Spd and Spm urinary levels were not detectable, while Pu increase in urine is the best biomarker to detect lymphoma growth. Furthermore, Pu urinary levels decreased significantly in mice treated intraperitoneally with B. fagaroides. In this model, variations of the Pu urinary level is an effective biomarker of neoplastic growth as it allows to follow the evolution of L5178Y lymphoma, providing an in vivo assay for the antitumoral effect of B. fagaroides and other drugs.

LAS POLIAMINAS COMO BIOMARCADORES DE LA ACTIVIDAD ANTITUMORAL DE Bursera fagaroides
Ramón Reynoso-Orozco, Anne Santerre, Jorge Iván Delgado-Saucedo, Josefina Casas Solís, Salvador Velázquez-Magaña and Ana María Puebla-Pérez

RESUMEN

En células normales y tumores las poliaminas (PAs) putrescina (Pu), espermidina (Spd) y espermina (Spm) son requeridas en múltiples funciones fundamentales del ciclo celular. Altos niveles de PAs han sido reportados en varios tipos de cáncer, por lo que han sido propuestas como biomarcadores del desarrollo tumoral. En el presente trabajo se reporta su utilidad como biomarcadores de la evolución del linfoma murino L5178Y en diferentes fluidos, células y tejidos. Los hallazgos también fueron aplicados al seguimiento del efecto antitumoral de Bursera fagaroides, ya reportado previamente. Se utilizó cromatografía de intercambio iónico para determinar los niveles de PAs en orina, células peritoneales, linfocitos circulantes, esplenocitos, mesotelio e hígado de ratones BALB/c a los 10, 17 y 24 días de evolución del tumor y de ratones tratados con el extracto hidroalcohólico de la corteza de B. fagaroides administrado por vía oral o intraperitonealmente (i.p.) Los niveles urinarios de Spd y Spm no fueron detectables, mientras que el incremento de Pu en orina es el mejor biomarcador del crecimiento del linfoma. Además, los niveles urinarios de Pu disminuyeron significativamente en ratones tratados intraperitonealmente con B. fagaroides, lo cual refuerza resultados anteriores. En este modelo la variación de los niveles urinarios de Pu es un biomarcador efectivo del desarrollo neoplásico, dado que permite seguir la evolución del linfoma L5178Y. Además, proporciona una herramienta para estudiar in vivo, el efecto antitumoral de B. fagaroides y de otros fármacos.

Introduction

Polyamines (PAs) such as putrescine (Pu), spermidine (Spd) and spermine (Spm) are ubiquitous cationic biomolecules necessary for completion of the cell cycle (Cohen, 1998). The enzymatic activity of ornithine decarboxylase (ODC), which is one of the limiting enzymes of PAs biosynthesis, is not well regulated in cancer cells, participating in the transformation of cell phenotype from normal to malignant (Babbar and Gerner, 2003). It has been demonstrated that ODC activity is elevated in tumors, leading to high levels of PAs in most types of cancer (Cañizares et al., 1999; Pegg et al., 2003). Intra and extra cellular levels of PAs, specially urinary PAs, are considered biomarkers of illnesses that...
AS POLIAMINAS COMO BIOMARCADORES DA ATIVIDADE ANTITUMORAL DE *Bursera fagaroides*

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**RESUMO**

Em células normais e tumoriais as poliaminas (PAs) putrescina (Pu), espermidina (Spd) e espermina (Spm) são requeridas em múltiplos funções fundamentais do ciclo celular. Altos níveis de PAs têm sido relatados em vários tipos de câncer, pelo que tem sido propostas como biomarcadores do desenvolvimento tumoral. No presente trabalho se relata sua utilidade como biomarcadores da evolução do linfoma murino L5178Y em diferentes fluidos, células e tecidos. As descobertas também foram aplicadas ao seguimento do efeito antitumoral de *Bursera fagaroides*, já relatado previamente. Utilizou-se cromatografia de intercambio iônico para determinar os níveis de PAs em urina, células peritoniais, linfócitos circulantes, exsudatórios, mesotelio e fígado de ratos BALB/c aos 10, 17 e 24 dias do desenvolvimento do tumor e de ratos tratados com o extrato hidroalcoólico da camada de *B. fagaroides* administrado por via oral ou i.p. Os níveis urinários de Spd e Spm não foram detectáveis, enquanto que o incremento de Pu na urina é o melhor biomarcador do crescimento do linfoma. Além disso, os níveis urinários de Pu diminuíram significativamente em ratos tratados intraperitonealmente com *B. fagaroides*, o qual reforça resultados anteriores. Neste modelo a variação dos níveis urinários de Pu são um biomarcador efetivo do desenvolvimento neoplásico, devido a que permite seguir a evolução do linfoma L5178Y. Além disso, proporciona uma ferramenta para estudar in vivo, o efeito antitumoral de *B. fagaroides* e de outros fármacos.

affect cell growth and proliferation (Jeevanandam and Petersen, 2001; Khuhawar and Qureshi, 2001). Moreover, these PAs, their biochemical analogs and several synthetic inhibitors of the enzymes responsible for their biosynthesis, are under study in alternative cancer treatment strategies (Huang et al., 2003). The hydroalcoholic extract of the medicinal plant *Bursera fagaroides* presents immunomodulatory activity and diminishes neoplastic growth in the BALB/c murine L5178Y lymphoma model. The extract is active at least until day 17, when administered via i.p. at 100mg/kg body weight for 15 days, but not when given orally or when the vehicle was administered alone (Puebla-Pérez et al., 1998).

In the present work, the levels of the three biogenic amines Pu, Spd and Spm were compared in healthy mice and mice with L5178Y lymphoma, in order to study the use of PAs as biomarkers of tumor growth and the effect of the hydroalcoholic extract of *B. fagaroides*.

**Materials and Methods**

*Bursera fagaroides*

The hydroalcoholic extract of *B. fagaroides* was prepared according to Puebla-Pérez et al. (1998) and administered either i.p. or orally (100mg/kg body weight) for a 15 days period, starting 24h after tumor cell inoculation.

**Animals**

BALB/c adult male mice haplotype H-2d of 6 to 8 weeks of age, 23-27g, were kept in polycarbonate cages in a controlled temperature room at 22°C and alternating 12h periods of light and dark. They were fed with a special diet for rodents (Purina-Mexico) and purified water ad libitum. The experiments were conducted under standard laboratory protocols.

**Study groups**

Seven study groups of five mice each were used. One group of healthy mice and six groups of mice inoculated with 2×10^5 L5178Y cells. Basal levels of PAs were detected at day 10 in healthy mice (“healthy” group). For the time-course determination of PAs levels, groups of mice were sacrificed at 10, 17 and 24 days.

Two groups of inoculated mice were treated with *B. fagaroides*. One group was injected i.p. while the other was given the extract orally. As a control group for this experiment, five mice were inoculated with L5178Y cells (“tumor” group). These three groups were sacrificed at day 17.

**Tumor cells**

The murine lymphoma L5178Y is a thymic haplotype H-2d tumor conserved by weekly i.p. transplants in BALB/c mice. The tumor is mortal and kills the mice in 15 ±2 days when inoculated via i.p. with 20×10^6 cells. In the present study 2×10^5 cells were inoculated (at day 0) to induce lymphoma growth.

**Chromatographic analysis**

Pu, Spd, and Spm separations were done in duplicates according to Villanueva et al. (1987) using HPLC and 1,7-diaminohexane (Sigma, St Louis, USA) as an external standard. The samples were centrifuged at 10000rpm for 20min at 4°C and finally frozen at -80°C until HPLC analysis.

**Peritoneum cells.** Mice were sacrificed in an ether chamber; the peritoneum was obtained by incision and washed with 0.5 ml of Hank’s balanced solution (HBSS; Sigma). The cellular suspension was centrifuged at 2500rpm for 5min. The supernatant was discarded and the cellular pellet washed twice with HBSS. Cells were resuspended (5×10^5 cells/80μl TCA-HCl), mechanically lysed, centrifuged at 10000rpm for 20min at 4°C and finally frozen at -80°C. At the time of HPLC, the sample was centrifuged (10000rpm for 2min) and 80μl of the supernatant analyzed.

**Circulating lymphocytes.** Mice were injected i.m. with a solution 200IU of heparin (PISA, Jalisco, Mexico) and sacrificed 30min later. The blood was obtained by heart puncture and the plasma and cellular package were separated by density gradient according to Boyum (1968). One million cells were placed in a microtube and centrifuged...
Putrescine (Pu) in urine and average weight as biomarkers of cancer growth in urine. BALB/c mice, healthy and during lymphoma growth.

Spermidine (Spd) levels in cells of BALB/c mice during lymphoma growth and control groups.

Spermine (Spm) in mesothelium and liver.

Statistical analysis

The experiments were analyzed using one-way ANOVA followed by pair wise multiple comparison procedures (Student-Newman-Keuls method). Friedman repeated measures analysis of variance on ranks (with the Sigma Stat version 2.03) and paired samples Student-Newman-Keuls test.

Results

Figure 1 shows that urinary levels of Pu were 10 times higher in mice with tumors, at days 10, 17 and 24, as compared to healthy mice. Spd and Spm were also considered, but the urine volume was insufficient for detection with the employed system.

As far as cells were concerned (Figure 2) Spd levels in circulating lymphocytes show a particular pattern and at day 17 and 24 are significantly higher in L5178Y mice than in healthy mice (Figure 2a). In spleenocytes, Spd levels rose at day 24 of tumor induction, when mice were visibly ill (Figure 2b). Peritoneal cells were not available until day 10 of tumor growth and Spd levels dropped at days 17 and 24 as compared to day 10 (Figure 2c).

Figures 3a and b present results of the determination of Spd and Spm in the mesothelium. It can be observed that, in this tissue, Spd and Spm are useful biomarkers of lymphoma growth, as their levels increased significantly at day 24 (Spd) and at days 10, 17 and 24 (Spm) of can-

Figure 1 Putrescine (Pu) in urine and average weight as biomarkers of cancer growth in urine. BALB/c mice, healthy and during lymphoma growth.

* ANOVA one way and Student-Newman-Keuls test (p<0.05) at days 10, 17 and 24 vs healthy group. Average weight for each group, n = 5.

* Friedman repeated measures analysis of variance on ranks, two-way interaction (p<0.0001).

Figure 2. Spermidine (Spd) levels in cells of BALB/c mice during lymphoma growth and control groups.

* One way ANOVA and Student-Newman-Keuls test (p<0.05) in a (days 17 and 24 vs healthy and day 10), b (day 24 vs healthy, days 10 and 17) and c (day 10 vs days 17 and 24).

Figure 3. Biomarkers in BALB/c mice tissues during lymphoma growth. a: spermidine in mesothelium, b: spermine in mesothelium and c: ornithine in liver.

* One way ANOVA and Student-Newman-Keuls test (p<0.05) in a (day 24 vs healthy days 10 and 17), b (healthy vs days 10, 17 and 24) and c (healthy and day 24 vs days 10 and 17).
cer growth, as compared to healthy organisms.

It is also of interest to note that the levels of ornithine, Pu precursor amino acid, decreased significantly at days 10 and 17 of lymphoma growth, compared to basal levels, and then increased at day 24 when mice were in terminal stages (Figure 3c).

The effect of the B. fagaroides treatment on Pu levels in the urine of mice with lymphoma, 17 days after L5178Y inoculation, is shown in Figure 4. Urinary Pu decreased significantly when the extract is administered i.p., but not so when given orally. Also, at least up to sacrifice day, the plant extract administered i.p. significantly slowed down lymphomas’ progression as the volume of the ascitic liquid did not increase much further, and lowered the levels of Pu in urine of mice with tumors. Likewise, the animals that showed tumor growth also presented >4g of weight increase (Figure 4).

Discussion

The comparative time-course analysis of the PAs levels measured in different tissue samples from healthy mice and mice with lymphoma, using ion exchange chromatography, was used to determine which PAs are better biomarkers of cancer growth in this model. This basic information provides a tool to screen in vivo the effect of natural compounds with demonstrated or potential antitumoral activity (Puebla-Pérez et al., 1998); in this particular case, from the hydroalcoholic extract of Bursera fagaroides.

The variation in Pu urinary level is the best biomarker in this experimental model, as Pu levels increased markedly in the urine of mice during lymphoma growth. Urine collection is inexpensive, easy, rapid and non-invasive (Gehrke et al., 1974; Meyskens and Gerner, 1999; Ochoa et al., 2002) and sample preparation for HPLC analysis is also fast and direct. However, the insufficient volume of urine obtained hindered Spd and Spm detection.

The levels of the triamine Spd increased in spleenocytes during lymphoma growth (Figure 2c), which could be due to the loss of control of PAs metabolism because of L5178Y cell proliferation within the peritoneum.

The mesothelium interacts with tumor cells and immune system cells, and participates in a series of molecular exchanges. For these reasons its study is of particular interest when analyzing the influence of drugs that could be directly applied i.p., as in the case of the natural extract used here. It can be observed (Figures 3a, b) that Spd and Spm are useful biomarkers of lymphoma growth as their levels significantly increase at day 24 (Spd) and at days 10, 17 and 24 (Spm) of cancer growth compared to healthy organisms. These results confirm those published by Milovic (2001) and Kobayashi et al. (2003) in other models. Milovic (2001) pointed out that during cancer growth Spd comes mostly from the diet, and Kobayashi et al. (2003) demonstrated that Spd and Spm, but not Pu, are the biologically active PAs. The diamine Pu is oxidatively deaminated by diamine oxidase (DAO) and converted to an important energy source through the Krebs cycle (Babbar and Gerner, 2003; Walters, 2003).

It is of interest that in the liver, PAs do not behave as biomarkers of tumor growth, as their levels do not show statistically significant differences (data not shown). Kitani and Fujisawa (1988) indicated that the liver is characterized by a low ornithine decarboxylase (ODC) activity, which could explain the small variation in PAs levels. However, the levels of their amino acid precursor, ornithine, provided a useful measurement, as it decreased significantly, compared to basal levels, at days 10 and 17 of lymphoma growth, and then increased at day 24, when mice were in a terminal stage.

The time-course analysis of PAs levels in different tissue samples from healthy mice and mice with lymphoma confirms its differential distribution (Pryme and Bardeau, 2001) and points out that Pu in urine is the best biomarker of cancer growth in the present experimental model. Variations of Pu levels in urine provided an easy biochemical tool to study in vivo the antitumoral activity of a series of natural and synthetic substances.

The effect of the treatment with a B. fagaroides extract on Pu levels in the urine of mice with L5178Y lymphoma, 17 days after inoculation, is shown in Figure 4. Pu levels decreased significantly when the extract is given i.p. but not orally, similar to the antitumor activity described by Puebla-Pérez et al. (1998). In addition, tumor growth is directly related to weight increase, due to ascites in the mice peritoneum and this time was not the exception. Probably, B. fagaroides has an inhibitory effect on ODC activity, but more studies are required to test this.

It was thus confirmed that, at least up to sacrifice day, the plant extract administered i.p. significantly slows down the progress of the lymphoma and lowers the urinary levels of Pu in the mice with the tumor. Further work is being planned to characterize the chemical composition of the B. fagaroides plant extract, which is rich in flavonoids and saponins (Puebla-Pérez et al., 1998), as several authors have found that ODC activity is sensitive to flavonoids (Bomser et al., 1996; Frydman and Valasinas, 1999; Middleton et al., 2000). Research is also underway to find out if the variation in Pu urinary levels is related to modulation of the ODC activity, as this is a limiting enzyme in PAs metabolism, and to monitor the enzyme activity in the same model, in order to determine if the antitumoral activity and low levels of urinary Pu could be due to the kidney control of PAs metabolism, in particular through the inhibition of the enzyme activity.

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