Early Administration of Peroxisomicine A1 (T-514 Extracted from *K. parvifolia* Seeds) Causes Necrosis of Implanted TC-1 Cells without Affecting Target Organs in a Murine Model

La Administración Temprana de Peroxisomicina A1 (T-514 Extraída de las semillas de *K. parvifolia*) Causa Necrosis de Células TC-1 Implantadas sin Afectar Órganos Blanco en un Modelo Murino

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SUMMARY: Peroxisomicine A1 (PA1), one of the toxins isolated from seeds of plants of the *Karwinskia* genus, whose targets organs are the liver, kidney, and lungs. There is a selective toxicity *in vitro* to cancer-cell lines derived from the lungs, liver, and colon, compared to normal cell lines. PA1 caused apoptosis in several cancer-cell lines in culture. In toxic doses to rodents, it causes extensive apoptosis in the liver, kidney, and lungs. In our study we were interested in evaluating, for the first time, the morphological effects of administration of PA1 to implanted TC-1 cells and in the target organs *in vivo*. The TC-1 cells were cultured and injected into the hind limb of C57BL-6 mice. The animals were divided into 3 groups; those treated with four doses of 1 mg/kg each of PA1, the untreated control, and the vehicle-control groups. All mice were killed 10 days after cell implantation. Samples were obtained from TC-1 cells at the implantation site and from the liver, kidney, and lungs. The samples were processed for examination under light and electron microscopy. In the PA1-treated group, the TC-1 cells had necrosis, whereas in the control groups the tumor cells were undamaged. The target organs did not show any lesions. We demonstrated for the first time that there is a selective toxic effect of PA1 on the TC-1 cells *in vivo*.

KEY WORDS: Antineoplasic effect; Necrosis; Peroxisomicine A1; Selective toxicity; TC-1 cells.

INTRODUCTION

Peroxisomicine A1 (PA1) or toxin T-514 is a dimeric anthracenone isolated from the endocarp of plants of the *Karwinskia* genus. It belongs to the group of toxic compounds identified by Dreyer *et al*. (1975) in these plants, and named according to their molecular weight as T-496, T-514, T-516, and T-544. Bermudez *et al*. (1986) reported that in rodents the target organs for T-514 are the lungs, liver, and kidney. Piñeyro *et al*. (1994) described for the first time the presence of the selective toxicity of T-514 on human cancer-cell lines derived from the liver, lungs, and colon compared to their normal counterparts. In the same study they compared the selective toxicity of several known cytotoxic drugs (vincristine, epidoxorubicine, and 5-fluoracil) with the effect of T-514. They noted that normal cells were more sensitive to the effect of the tested drugs than tumor cells, whereas tumor cells were more sensitive to T-514 than normal cells. These findings led them to carry out further experiments to suggest T-514 as a potential antineoplasic drug. Piñeyro (1995; 1996) obtained a patent for the use of T-514 as a potential antineoplasic agent from the European Economic Community, USA, Japan, Korea, Canada, and Mexico.

Preliminary data about the mechanism of action of T-514 indicates that the peroxisome is the target organelle in yeast (Sepúlveda-Saavedra *et al*., 1992) and in rodents (Moreno-Sepulveda *et al*., 1997, Sepúlveda-Saavedra *et al*., 1998). For this reason the compound was renamed peroxisomicine.

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Lansiaux *et al.* (2001) reported the presence of apoptosis caused by T-514 in human leukemia cells *in vitro*. Martínez *et al.* (2001) demonstrated the presence of apoptosis in several transformed cell lines *in vitro* when treated with PA1, such as HeLa, mammary, colon adenocarcinoma, and hepatoma cell lines. When given to normal nontumor-bearing mice in high toxic doses (28 mg PA1/kg body weight) *vs* 1 mg PA1/kg body weight given in our study, T-514 caused extensive apoptosis in the target organs, liver, kidney, and lungs (Soto-Domínguez *et al.*, 2005). Preliminary results showed that mice treated with PA1 had a decrease in tumor growth compared to the control groups and an increase in survival time, which studies were made in our laboratory. The purpose of our study was to evaluate the morphological effect of an early administration of low doses of T-514, ten days post-implantation of TC-1 cells when no tumor can yet be detected, and to compare this with the effects in the liver, kidney, and lungs.

MATERIAL AND METHOD

Tumor cell-line TC-1. The TC-1 cell-line was obtained from ATCC (CRL-2785) (Manassas, VA, USA). According to Lin *et al.* (1996) this cell line was derived from a primary culture of epithelial cells of the mouse strain C57BL-6 (Lin *et al.*). When these cells are implanted in the C57BL-6 mice, there is no immunological rejection and these mice were then successfully used as a model of murine cancer (Lin *et al.*; Greenstone *et al.*, 1998; Tuve *et al.* 2007).

Cell-culture techniques and cell implantation. Cells were cultured in Dulbecco´s modified Eagle Medium-1 supplemented with 10% fetal bovine serum, incubated at 37°C in 5% CO_2 and 95% humidity. At confluence, the cells were harvested with 0.25% trypsin. The cell suspension was maintained in ice, washed with phosphate saline buffer (PBS), pH 7.2 - 7.4. The cells were adjusted to a final concentration of 5 x10^4/100 mL of PBS. This amount was implanted in the subcutaneous tissue of the hind limb of male mice strain C57BL-6, weighing 25 to 30 g (Harlan, Mexico). Before and after injection, cell viability was determined with trypan blue yielding a value of >95% viability. The animals were kept under standard laboratory conditions of temperature 22 to 25 °C, 65% to 70% humidity, fed with a standard diet and water *ad libitum*, with 12-h light-dark cycles in the animal facilities of the Histology Department, Facultad de Medicina, UANL.

Peroxisomicine A1. PA1 (toxin T-514) was purified and identified by Ramírez-Durón and Waksman de Torres from fruits of *K. parvifolia* using procedures already described (Osorio-Perez *et al.*, 2003; Fernández-Ramírez *et al.*, 2004).

Experimental groups. Eighteen mice strain C57BL-6 were used and divided into three experimental groups; the PA1-treated group (n = 6), a vehicle-control group (n = 6), and an untreated-control group (n = 6). All animals received a subcutaneous injection of a 5 x 104 TC-1-cell suspension into the hind leg. The day of the injection was day 0. The PA1-treated group received, 4 doses of 1 mg/ kg body weight of PA1 in carthamus oil ip, on days 2, 4, 6, and 8 post-implantation. The vehicle-control group received only carthamus oil on the same days, last group received no treatment (absolute control).

Tissue collection. To evaluate the morphological effects, samples were collected at day 10 after cell implantation, at which time no tumor was present. All animals were killed by cervical dislocation. Tissue samples were collected from the hind leg at the site of the cell implantation and from the lungs, liver, and kidney.

Morphological analysis. All mice were perfused through the left ventricle with a fixative solution of 0.25% glutaraldehyde in cacodylate buffer, pH 7.2 to 7.4, samples were collected and further processed either for light microscopy or for electron microscopy.

Light microscopy. The samples were fixed in a freshly prepared solution of 4% paraformaldehyde dissolved in PBS, processed by routine histological techniques, and embedded in paraffin. Five-micron sections were obtained, mounted, and stained with the hematoxylin-eosin technique or with the Masson trichromic-method (Masson, 1929).

Electron microscopy. Samples were quickly removed and placed in a drop of fixative, minced, and then transferred to a vial containing 4% paraformaldehyde, 5% glutaraldehyde, and 0.05% picric acid in 0.1 M PBS (Ito & Karnovsky, 1968). After one hour the tissues were rinsed briefly in buffer and postfixed in 2% aqueous osmium tetroxide for one hour at room temperature. The tissues were then treated with a 1% aqueous solution of uranyl nitrate. After rapid dehydration with ethanol, the tissues were embedded in epoxy resin using the technique of Luft (1961). Sectioning was done with a diamond knife on an RMC ultramicrotome. The sections were collected on 200-mesh copper grids without a supportive substrate, stained with uranyl acetate (Watson, 1958), and then by lead citrate (Venable & Coggeshall, 1965). Microscopy was done in a Carl Zeiss EM10 electron microscope. One-micron sections were mounted on glass slides and stained with 1% toluidine blue for study with the light microscope.

TUNEL test for the demonstration of chromatin fragmentation. For the TUNEL test (Gavrieli *et al.*, 1992)

the kit, TACS 2TdT *in situ* Apoptosis Detection, Trevigen, was used. The kit instructions were followed and samples were analyzed by light microscopy.

Immunohistochemistry and quantitative analysis of identified cell populations. For cell identification, specific monoclonal antibodies (Dako Cytomation, Inc.) were used to identify T-lymphocytes anti-CD3+, B-lymphocyte anti-CD20+, macrophage anti-CD15+, and NK cell anti-CD56+. The procedures were done using the kit instructions. After identification of infiltrating cell types, positive cells for each marker were counted in three slides (one for each mouse) of each group. Briefly, a first field was selected at random with the oil immersion objective (100 X) and in the eight consecutive fields cells were counted. The total number of cell types was counted. The percentage of positive cells was calculated and an average value was obtained, as was the standard deviation and standard error for each group. Data were graphed and a statistical Student's t-test was used with a degree of significance of P<0.05.

RESULTS

Morphological observations of TC-1 cells. In animals treated with PA1, the histological analysis of the cell-implantation site showed necrosis of TC-1 cells, with cell debris and the remains of pyknotic nuclei from the tumor cells. The normal tissues surrounding tumor cells were undamaged (Fig. 1A). These features were confirmed at the ultrastructural level, where tumor cells were seen with the rupture of the plasmalemma, cytoplasm loss, swollen mitochondria with matrix loss, and pyknotic nuclei with the rupture of the nuclear envelope (Fig. 1B). The untreated and vehicle-control groups showed the integrity of the tumor cells. The TC-1 cells were found mingled with the connective tissue around muscle fibers. The muscle fibers showed an important decrease in size and an irregular shape. The tumor-cell nuclei were seen in the middle of the fiber cytoplasm (Fig. 1C). The muscle fibers also showed a change in the staining pattern with the Masson method, instead being the bright red of normal muscle fibers (Fig. 1D), a blue staining pattern was seen (Fig. 1E).

Mixed with the TC-1 cells were several types of infiltrating, mononuclear cells, these observations were confirmed with the immunohistochemistry techniques used.
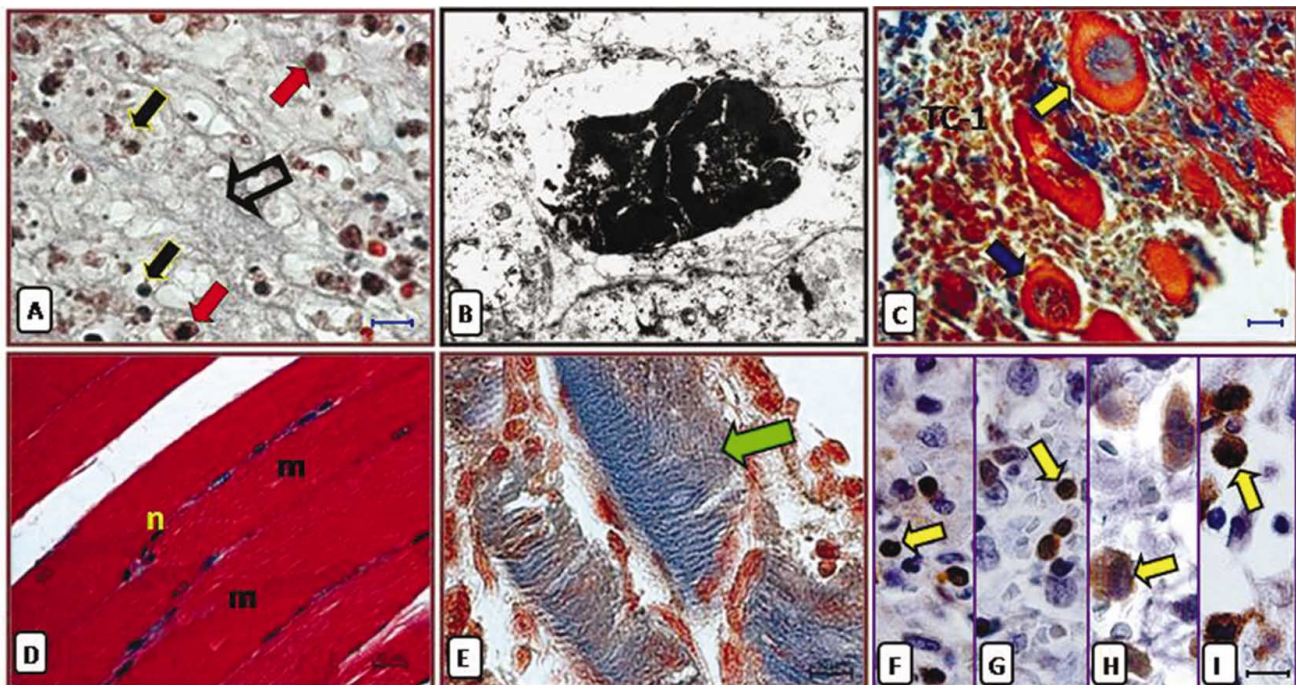

Fig. 1. A) TC-1 cells at the implantation site, PA1 treated. Light micrograph: cell necrosis (empty arrow), pyknotic nuclei (black arrows), and mononuclear cells (red arrows). Paraffin embedded, Masson staining. 10-μm bar. B) TC-1 cells at the implantation site, PA1 treated. Electron micrograph. Cell with signs of necrosis, observe pyknotic nuclei. 10,000 x. C) TC-1 cells at the implantation site, Untreated group. Light micrograph: TC-1 cells (TC-1), skeletal muscle fibers with nucleus in a central location (blue arrow). Fibers with change in staining pattern (yellow arrow). Paraffin embedded. Masson stain. 20-μm bar. D) Normal skeletal muscle cells (m) stained with Masson technique, observe bright red color, peripheral nuclei (n). Paraffin embedded. Masson stain. 20-μm bar. E) TC-1 cells at the implantation site, untreated group. Light micrograph: The micrograph shows the color variation in skeletal muscle fibers (green arrow). Paraffin embedded. Masson stain. 10-μm bar. F, G, H, I) TC-1 cells at the implantation site, PA1 treated. Light micrograph. Observe positive cells

The cell types identified were T-lymphocytes CD3+ (Fig. 1F), B-lymphocytes CD20+ (Fig. 1G), macrophages CD15+ (Fig. 1H), and NK cells CD56+ (Fig. 1I).

Morphological observations of target organs. All samples from the liver, lungs, and kidney, including that treated with PA1, showed normal histological features. No signs of toxicity were seen in the treated animals. These features were confirmed by using the electron microscope, where no abnormal signs were observed in any sample of the target organs, either treated with PA1 or untreated (Figs. 2A to 2F).

TUNEL technique. The TUNEL reaction was negative for chromatin fragmentation in all samples evaluated (Figs. 2G to I).

Infiltrating cell quantification. Cell counts for each identified cell type and experimental group demonstrated the presence of a pattern in which the largest percentage of infiltrating cells was T-lymphocytes (17.3% ± 0.4%) in all groups. Macrophages (13.0% ± 0.3%) were the second most abundant cells, then B-lymphocytes (10.8% ± 0.4%), with the least frequent NK cells (10.3% ± 0.3%). The amounts of the infiltrating cells present in each group did not show differences among groups, either treated with PA1 or untreated (Fig. 3).
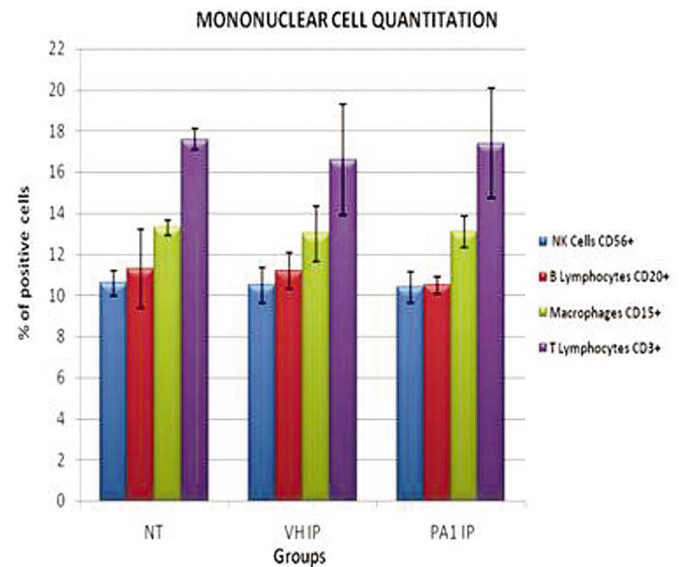

Fig. 3A) Graphic of quantization of mononuclear cells. Cell counts for each identified cell type and experimental group demonstrates the presence of a pattern where the largest percentage of infiltrating cells is T-lymphocytes in all groups. Macrophages were the second most abundant cells, and then B-lymphocytes, with the least frequent the NK cells. Amounts of the infiltrating cells present in each group did not show differences among groups, either treated or untreated with PA1
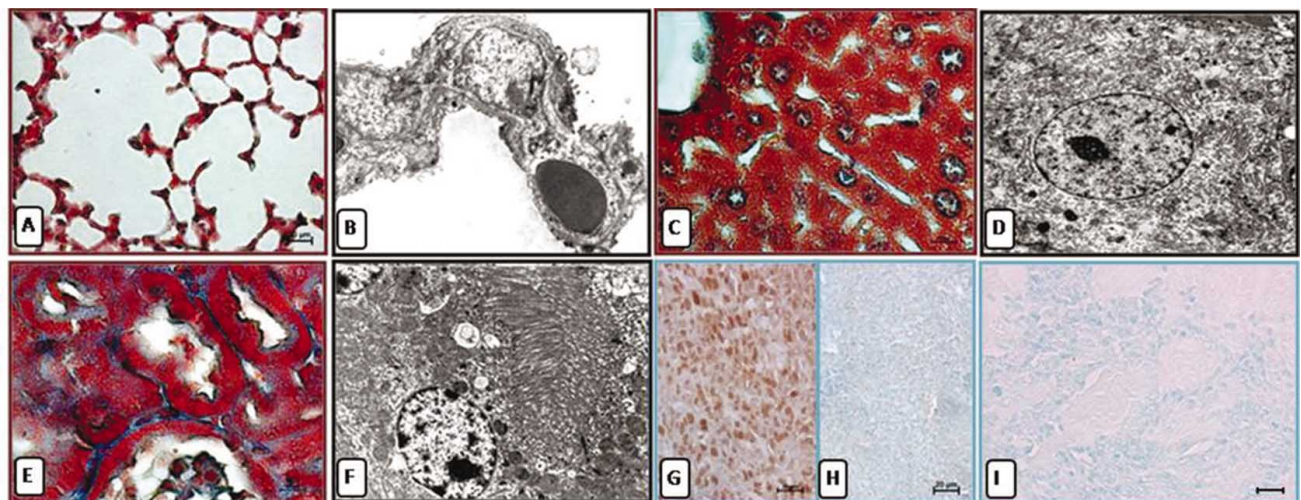

Fig. 2. A) Lung, PA1 treated. Light micrograph: Normal histological image. Paraffin embedded, Masson staining. 20-µm bar. B) Lung, PA1 treated. Electron micrograph: Observe normal appearance. 3,150x. C) Liver, PA1 treated. Light micrograph: Observe normal histology. Paraffin embedded, Masson staining. 20-µm bar. D) Liver, PA1 treated. Electron micrograph: No lesions are present. 6,150x. E) Kidney, PA1 treated. Light micrograph: Observe normal histology. Paraffin embedded, Masson staining. 20-µm bar. F) Kidney, PA1 treated. Electron micrograph: No lesions are present. 6,150x. G) TUNEL Positive control. Tumor mass. Untreated group. Light micrograph: observe brown staining in positive nuclei. Paraffin embedded. TUNEL. 20-µm bar. H) TUNEL Negative control. Tumor mass. Untreated group. Light micrograph: observe negative nuclei. Paraffin embedded. TUNEL. 20-µm bar. I) TC-1 cells from the implantation cell site, PA1 treated mice. Light micrograph: Negative nuclei for chromatin fragmentation. Paraffin embedded. TUNEL. 20-µm bar

DISCUSSION

The results of our study demonstrate for the first time that there is a selective toxicity *in vivo* of PA1 on the TC-1 cells implanted into mice, because they show necrosis whereas the target organs, the lungs, liver, and kidney, do not show any lesions.

The histological and ultrastructural study of the TC-1 cells showed the presence of signs indicative of necrosis; cell membrane rupture, cytoplasm extraction, loss of organelles, and pyknotic nuclei. No morphological signs indicative of apoptosis were present, such as apoptotic bodies or characteristic chromatin condensation. The TUNEL technique to demonstrate chromatin fragmentation was negative in all samples tested, except in the positive-test control slides treated with nuclease. These findings are different from those reported in other studies made on leukemia cells (Lansiaux *et al*.), where they reported a dose-dependent apoptosis caused by PA1. There is also a report of apoptosis caused in HeLa cells, and in mammary adenocarcinoma, colon adenocarcinoma, and hepatoma (Martínez *et al*.). The differences in the results reported *in vitro*, compared with the study *in vivo* reported here, could be caused by the concentration of PA1 reaching the cells, which could be greater in the system *in vitro* than in the model *in vivo*. In the report of Lansiaux *et al*. the low concentrations of PA1 did not cause apoptosis, whereas in normal nonbearing-tumor mice intoxicated with high doses of PA1 (28 mg of PA1/kg body weight) extensive apoptosis is present in the liver, kidney, and lungs (Soto-Dominguez *et al.*). The actual mechanism behind the different sensitivity among normal and tumor cells to PA1 is unknown.

For the types of lymphoid cells present, we observed the same pattern in each sample evaluated, both treated and untreated with PA1. This indicates that PA1 is not related to the presence of these cells, rather their presence could be caused by the recognition of membrane antigens present in the TC-1 cells, namely the oncoproteins E6 and E7 of the Human Papilloma Virus-16 (Lin *et al*.). The lymphoid cell counts demonstrate that there is a predominance of T lymphocytes in all groups, followed by macrophages, B lymphocytes, and NK cells, suggesting a cellular immune-response directed against cell antigens.

The target organs did not show any toxic effect, which correlates with the findings obtained in the doses-response graph, showing that mortality begins when 10 mg of PA1/body weight are administered. Thus, it is likely that larger doses of PA1 could be administered to improve the beneficial results found in this study. It is feasible to increase the dose as long as no toxic effects are detected in the target organs. Further experiments are being made in our laboratory to test this possibility, to determine if treated tumor-bearing mice show a longer survival time when treated with PA1, and to evaluate the rate of tumor growth in treated *vs* untreated mice. Preliminary observations obtained by our group indicate that this true.

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RESUMEN: Peroxisomicina A1 (PA1), una de las toxinas aisladas de las semillas de plantas del género *Karwinskia*, cuyos órganos blanco son hígado, riñón y pulmón. Hay una toxicidad selectiva in vitro contra líneas celulares cancerosas derivadas de pulmón, hígado y colon, comparadas con líneas celulares normales. PA1 causa apoptosis en varias líneas celulares malignas en cultivo. En dosis tóxicas a roedores, causa extensa apoptosis en hígado, riñón y pulmón. En nuestro estudio, estuvimos interesados en evaluar por primera vez los efectos morfológicos de la administración temprana de PA1 sobre células TC-1 implantadas y los órganos blanco *in vivo*. Las células TC-1 fueron cultivadas e implantadas en la extremidad posterior de ratones C57BL-6. Los animales fueron divididos en tres grupos: tratado con cuatro dosis de 1 mg/kg de peso de PA1, control sin tratamiento y control vehículo. Todos los animales fueron sacrificados 10 días posterior al implante de las células. Se colectaron muestras del sitio del implante de las células TC-1 y de hígado, riñón y pulmón. Las muestras fueron procesadas para su análisis a microscopía de luz y microscopia electrónica de transmisión. En el grupo tratado con PA1, las células TC-1 presentaron necrosis, mientras que en los grupos control las células tumorales se observaron sin daño. Los órganos blanco no mostraron lesión alguna. Demostramos por primera vez que existe un efecto tóxico selectivo de PA1 sobre las células TC-1 *in vivo*.

PALABRAS CLAVE: Efecto antineoplásico; Necrosis; Peroxisomicina A1; Toxicidad selectiva; células TC-1.

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