Expression of caspase 1 and histomorphology of lung after cladribine treatment

Marta Lis-Sochocka, Patrycja Chylinska-Wrzos, Ewelina Wawryk-Gawda, Barbara Jodlowska-Jedrych

Department of Histology and Embryology with Experimental Cytology Unit, Medical University of Lublin, Poland

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Address for correspondence
Marta Lis-Sochocka
E-mail: martasochocka@wp.pl

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Abstract

Background. Cladribine is a useful immunosuppressive drug for the treatment of autoimmune diseases, leukemias and multiple sclerosis (MS). Despite the drug having low toxicity, side effects have been reported connected with myelosuppression, neutropenia and severe anemia.

Objectives. The objective of this study was to investigate the influence of cladribine on lung pathomorphology and the expression of caspase 1 using immunohistochemistry method.

Material and methods. The study was conducted on Wistar rats, which were divided into: one control group (C) and one experimental group (E). In group C, the rats were given a 0.9% NaCl solution by way of a subcutaneous injection, at the same dose as the dose of drug used in the experiment. In group E, the animals received cladribine at a dose of 0.07 mg/kg/24 h by way of a subcutaneous injection. The animals were decapitated 24 h following the last dose. To detect collagen deposition, we utilized Masson’s trichrome staining. To evaluate the intensity of the inflammatory process in the lung, an immunohistochemistry reaction was carried out with the use of caspase 1.

Results. In group E, we observed an increase in the thickness of space between the alveoli. A statistically significant (p < 0.017243) difference between the thicknesses of the interalveolar septum was seen between the research groups. In E group, we observed regions with collagen deposition, alveolar epithelial cell hyperplasia, hyperemia and inflammatory cell infiltration. Caspase 1 activity was higher in the group E. The immunohistochemical reaction with caspase 1 was positive in 49% of all the interalveolar cells in the group E; however, in group C about 13% of the interalveolar cell showed positive immunohistochemistry (IHC) response.

Conclusions. Cladribine-based therapy might have negative influence on lung morphology. The interstitial changes in the lung tissue suggest that cladribine is a drug that may be the cause of drug-induced lung disease and may lead to several respiratory disorders.

Key words: inflammation, lung, caspase 1, cladribine
Introduction

Lungs are composed of very sensitive tissue. Their large surface has contact with many substances from the environment and with substances that are used incorporeally. Indeed, the list of drugs which can be the cause of lung toxicity is long. Furthermore, the clinical, radiological and histological symptoms are nonspecific; therefore, the diagnostic and recognition of drug-induced lung disease is difficult.1

Cladribine (2-CdA), a purine analogue which acts as antineoplastic and immunosuppressive agent, has been used in the treatment of malignances, autoimmune and degenerative diseases. The drug, 2-CdA, is a deoxycytidine analogue which is used in experimental multiple sclerosis (MS) treatment as it exhibits selective toxicity relative to lymphocytes and monocytes.2 According to the study undertaken by Leist and Weisert, the administration of the oral form of 2-CdA abates the number of lymphocytes for up to 6–12 months after the end of treatment.3 What is more, although 2-CdA mainly negatively affects the number of CD4+/CD8+ lymphocytes, it has been seen particularly as being effective in minimizing the CD4+ class.3–5 Cladribine is considered to be of relatively low toxicity in therapeutic doses, and any side effects have been seen only in the first 14 days of therapy or in the first month of treatment. In individual studies, the drug is generally well-tolerated by MS patients. Reported side-effects are connected with myelosuppression, neutropenia and severe anaemia, and with the complications that come about as a result of opportunistic infections. Infections of upper respiratory tract, infections of urinary tract, herpes zoster and HBV virus infections are among the most commonly reported side effects. In addition, patients have complained about general disorders such as fever, lack of appetite, tiredness, chills and general weakness. These clinical symptoms can be brought about by mucosa damage.2,4,6

Cladribine could also destroy healthy, functional cells, especially those which are rapidly dividing (the mucosa cells of the digestive system, the bone-marrow cells or the skin cells).7 Previous studies have shown that cladribine could initiate apoptosis in the epithelial cells covering the ovary, the uterine epithelial cells, the fallopian tube epithelium and in the epidermal cells.8–12 Literature reports suggest that patients may be susceptible to opportunistic infections during cladribine treatment due to the reduced number of immune system cells.

Multiple sclerosis is classified as an autoimmune disease, and autoreactive T cells, mainly of the CD4+ type, are involved in the development of it.13–15 Herein, activated T cells penetrate the blood–brain barrier and permeate to the central nervous system. Here, they stimulate other immune cells such as the macrophages. As a result of these multidirectional immune responses, foci of demyelination in white matter and the degeneration of axons will be seen.16,17 Moreover, the resulting errors that come about in the apoptosis and which interfere with the destruction of cytotoxic T cells also play an important role in MS pathogenesis.16 Immunomodulatory and immunosuppressive therapies have been commonly applied in MS treatment.18 In these therapeutic methods, certain oral anti proliferative drugs are prescribed, while other medicaments are being researched.19–22

Considering the results of our previous studies, and because there is no data on the impact of 2-CdA on the histology of the lung, the aim of the authors’ study is to ascertain the effect of 2-CdA on the lung pathomorphology. In our study, we used cladribine at a dosage that is normal for treating MS in people. Our intent is ascertain whether 2-CdA at this dose may be the cause of interstitial lung disease. In addition, by way of employing the immunohistochemistry method, we intend to study caspase 1 expression (a marker of inflammation).

Material and methods

The study was conducted on 10 female white Wistar rats (weighing about 250–300 g each), which were placed within one control group (C) and one experimental group (E), 5 animals in each. The animals were kept according to ethical standards set out by the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the European Community Council Directive of 24 November 1986, for Care and Use of Laboratory Animals (86/609/EEC), and accepted by the local ethics committee (Medical University of Lublin, Poland). Furthermore, consent no 126/2001 was obtained from the local bioethical committee.

In group E, the animals received cladribine (Biodribin; Institute of Biotechnology and Antibiotics, Warszawa, Poland) at a dose of 0.07 mg/kg/day by way of a subcutaneous injection for 6 successive days (in the morning), in 3 courses, with 5 weeks break between each (such scheme of dosage is normal for treating MS in people).23,24 In group C, the rats were given a 0.9% NaCl solution by way of a subcutaneous injection at the same dose as the dose of drug used in the experiment.

The animals of both groups were decapitated 24 h following the last injection.

During the experiment, the rats resided in cages (Techniplast17; Techniplast Kukuczka, Ustroń, Poland) of 0.5 m² area. They were fed granulated fodder (LSM; AnimaLab, Poznań, Poland) and had free access to normal water. The air humidity was 50–60%, the temperature of their environment was 20 ± 1°C, and a 24 h cycle was kept (12 h day, 12 h night). All stress factors were reduced to a minimum. In the time period of the experiment, we saw no symptoms of infection.

At the end of experiment (89 days), the animals were killed by decapitation, and lung samples (about 1 cm² area) were taken for histological and immunohistochemical examination.
For a histological investigation, the material was fixed in Baker’s solution, after which they were dehydrated in alcohol with increasing concentration, placed in xylene and put in paraffin. The paraffin blocks were cut utilizing a rotational microtome Leica (Leica Biosystems, Nussloch, Germany) RM 2135, in 5 µm sections, and the prepared samples were colored with hematoxylin and eosin (H+E). To detect collagen deposition, we utilized Masson’s trichrome staining. To evaluate the intensity of the inflammatory process in the lung, an immunohistochemistry reaction was carried out by way of using primary antibodies (Sigma®; Sigma-Aldrich, Poznań, Poland). In this, caspase 1 (ANTI-CASP1, HPA008936-100UL, antibody produced in rabbit, dilution: 1:50) was employed. The IHC study was conducted via the indirect immunoperoxidase method. In this way, 5 µm thickness paraffin fragments were placed on slides (Polysine®; VWR International, Poznań, Poland) and incubated all night at a 58ºC temperature. Next, the specimens were dehydrated with increasing concentrations of ethyl alcohol, and then immersed in xylene. Following this, the activity of the endogenous peroxidase was blocked for 5 min by way of immersion in a 3% hydrogen peroxide in methanol solution. Afterwards, the slides were rinsed in distilled water and subsequently in a solution of TBS. Next, the places of active antigen were unmasked by applying a thermal process. Herein, specimens were microwave (800 V) while in a 0.01 M citrate buffer, pH 6.0, by way of 3 x 5-min cycles. After washing in distilled water, the samples were then incubated with inactive serum (normal diluted serum) for 30 min to mask all places which could connect with caspase 1. Following a rinsing in a TBS solution, the samples were incubated for 24 h at 8°C, with the primary antibodies. To make the reaction visible, Novolink™ Polymer Detection Systems (Leica Microsystems, Wetzlar, Germany) was used. After this, the samples were re-rinsed in a solution of TBS and mottled by chromogen – 3-3’-diaminobenzidine (Novolink DAB; Vector Laboratories Inc., Burlingame, USA), which indicated a positive reaction by turning the brown. Finally, the cell nuclei of the samples were stained through the application of Mayer’s hematoxylin. A negative control was put into place for all sections of all the groups by the same method. This was without the primary antibody.

The study’s material was evaluated using an Olympus BX41 with digital camera image DP25 (Olympus, Tokyo, Japan). Calculations were made in Cell^D (Olympus, Tokyo, Japan).

In both research groups, 40 measurements of the thickness of the interalveolar septum were made in different view areas.

To assess the intensity of the immunohistochemistry reaction in the alveolar interstitial cells, different areas of ×400 vision were selected, and 300 cell-counts were analyzed. The places of different intensity of immunohistochemistry reaction were reported as: 1 (+) – a weak reaction, 2 (++) average response and 3 (+++) – strong reaction. Areas with a negative response were marked as 0 (–).

The statistical results surveys were analyzed in STATISTICA v. 10.0 (StatSoft Polska, Kraków, Poland).

The statistical differences of intensity of caspase 1 expression between examined groups were assessed with the Kruskal-Wallis nonparametric test. In addition, statistical correlations between the thicknesses of the interalveolar septum, and between the research groups, were calculated utilizing Student’s t-test. An overall p-value of less than 0.05 was considered to show a statistically significant result.

**Results**

**Hematoxylin and eosin stain**

In the control group (C), specimen tissue showed visible bronchioles of a different diameter which were easily differentiated as respiratory bronchioles, alveolar ducts and alveoli (Fig. 1). The alveoli are lined by 2 types of cells: pneumocytes type I (squamous) and pneumocytes type II...
Between the alveoli, were thin bands of connective tissue and blood vessels (Fig. 2). The mean value of the thickness between the alveoli was 6,312 µm, with the maximum and minimum value of 2.50 and 16.11 µm, respectively (Fig. 3). In the experimental group (E), we found evidence of nonspecific interstitial pneumonia (NSIP). A significant increase in the thickness of alveolar spaces was observed, and leukocytes, as well as macrophages and eosinophils, were detected in these spaces (Fig. 4). Inflammatory infiltrates with a predominance of lymphocytes were also recognizable around the blood vessels (Fig. 5). Furthermore, a mild hyperplasia of type II pneumocytes was observed. The mean value of the thickness of the interalveolar septum was 8.367 µm, while the minimum and maximum thicknesses were 2.04 µm and 21.40 µm, respectively. A statistically significant (p < 0.017243) difference between the thicknesses of the interalveolar septum was seen between the research groups.

Masson’s trichrome staining

In the experimental group (E), we observed regions with collagen deposition in their lung tissue, especially in the alveolar septa. What is more, alveolar epithelial cell hyperplasia, hyperemia and inflammatory cell infiltration were found (Fig. 6, 7).

The immunohistochemical study

Regarding group C, the immunohistochemical reaction with caspase 1 was negative in most interstitial cells (over 87%) (Fig. 8, 9). Moreover, only a few interalveolar cells showed a weak (8% of all cells) or a moderate (4% of all cells) IHC response. A strong expression of the caspase 1 protein, assessed as 3 (+++), was only visible in 0.67% of all the observed cells (Fig. 10). However, in group E, a positive, cytoplasmic immunohistochemical reaction was seen in 49% of all the interalveolar cells. Of this, more than 30%...
of the cells were assessed 1 (+) as revealing a weak reaction, while more than 16% of all cells showed an average of 2 (++) and 2% of the cells showed a strong 3 (+++) reaction. In the remaining cells (51%), the reaction was negative or at trace amounts (Fig. 9, 10). Of note: the difference in the caspase 1 expression between the control and experimental groups was statistically significant (p < 0.000001).

Discussion

Cladribine, as an antineoplastic and immunosuppressive agent, induces apoptosis in the cells and penetrates into the cells through the cellular membrane by way of nucleoside transporters. Within the cell, 2-CdA is converted into an active 2-CdATP metabolite. Its function in the cell leads to a series of enzymatic and structural changes, which affect the stability of damaging factors and repair mechanisms. Cladribine acts on proliferating and non-proliferating cells; its cytotoxic effect is also multidirectional. Although this drug is considered to have low toxicity, it is not completely safe, yet previous reports put forward the claim that cladribine is not associated with lung toxicity. Cladribine, like other purine analogues, is an antimetabolite, which exhibits selective toxicity with respect to lymphocytes and monocytes. In therapeutic doses, 2-CdA is well tolerated by patients, but some side-effects have been noticed. Betticher et al. reported that the main side effects observed in cladribine therapy were myelotoxicity and immunosuppression with regard to opportunistic infections (14%) such as pneumonia. Similarly, Van Den Neste et al. also reported that 42% of patients treated with 2-CdA and cyclophosphamide...
developed opportunistic infections, e.g. pneumonia caused by varicella-zoster virus.\textsuperscript{30} Furthermore, Montillo et al. observed pneumonia due to Enterococcus or cytomegalovirus in patients receiving cladribine at 4 mg/m\textsuperscript{2}/day and cyclophosphamide at 350 mg/m\textsuperscript{2}/day.\textsuperscript{31} In addition, Okawa et al. reported pneumonia induced by Cryptococcus after 2-CdA treatment. In this study, cladribine was administered in 4 courses.\textsuperscript{32}

Fridrik et al. evaluated the performance of 2-CdA in the treatment of patients with advanced non-Hodgkin’s lymphoma. In this study, patients received cladribine in a dose of 0.12 mg/kg intravenously daily for 5 days. This schema was repeated every 28 days for 4 cycles. During the treatment, certain side effects were observed, mainly hemato logical toxicity and respiratory system infections.\textsuperscript{33} Feenstra et al. also reported pulmonary toxicity (with a diffuse interstitial pneumonitis and with hypoxaemic respiratory failure) after the first course of cladribine therapy of non-Hodgkin’s lymphoma.\textsuperscript{34} In order to assess the possible changes that could occur in the lungs after the application of 2-CdA, in our work, at first, we carried out routine histological H+E staining. In this, lung histological examination of the control group showed normal pulmonary histology. What is more, the thickness between alveoli varied between 2.50 and 16.11 µm, with the mean value of the thickness being 6.312 µm. In the experimental group, however, an increase in the thickness of alveolar spaces was observed. The mean value of the thickness of interalveolar septum was 8.367 µm, while the minimal thickness was 2.04 µm, and the maximal was 21.40 µm. Of note: a statistically significant (p < 0.017243) difference between the thickness of the interalveolar septum was evident between the research groups. Moreover, inflammatory infiltrates with a predominance of lymphocytes were detected around the blood vessels. In addition, a fibrotic broadening of the alveolar septa was seen, and type II pneumocyte hyperplasia was observed. We put forward that the results of H+E staining is indicative of nonspecific interstitial pneumonia. This would be consistent with the findings of other authors who described the existence of pneumonia in patients treated with 2-CdA.

In order to confirm the inflammatory reaction in lung tissue, we conducted immunohistochemistry using caspase 1, which is a major marker of inflammation.

Caspase 1 belongs to the family of cysteine proteases. In the cytosol of a cell, this enzyme is synthesized as the inactive zymogen pro-caspase 1. Caspase 1 is activated as a response to inflammation, and it is believed that it is associated with dimerization and the autoproteolysis of enzymes. With time, characteristic large and small subunits (p20 and p10) of active caspase 1 are formed.\textsuperscript{35–37} In the first step of activation, in association with the NLR or PYHIN protein families, it forms the inflammasome complex. Proteins NLR/PYHIN are sensitive to pathogens, toxins and several sorts of infections.\textsuperscript{35} Activated caspase 1 initiates the secretion of pro-inflammatory cytokines such as interleukin 1β and 18.\textsuperscript{35,38} Our work found that in the control group C, in most interstitial cells (over 87% of all cells), the immunohistochemical reaction with caspase 1 was negative. In addition, a weak or an average IHC response (respectively, 8% and 4%) was seen in the examined cells. However, in experimental group E, a positive, cytoplasmic immunohistochemical reaction was seen in 49% of all interstitial cells. In control group C, in most interstitial cells (over 87% of all cells), the immunohistochemical reaction with caspase 1 was negative. In addition, about 30% of such cells presented a weak reaction, 16.3% of the cells showed an average reaction, and 2% of the cells reacted strongly. The dissimilarity in the occurrence of the caspase 1 expression between 2 groups: the control and the experimental, was, hence, statistically significant p < 0.000001. The results confirm the H+E staining observation.

Inflammatory reaction is the initial response to the lung injury. Activated inflammatory cells such as the neutrophils and the macrophages accumulate in the tissue and release harmful amounts of reactive oxygen species, as well as some pro-inflammatory cytokines that (in turn) activate collagen production in alveolar fibroblasts. The increasing amounts of matrix proteins distort the normal lung architecture and affect gas exchange.\textsuperscript{39} The interstitial changes in the lung tissue observed in our study suggest that cladribine is a drug that may be the cause of drug induced lung disease and may lead to several respiratory disorders.

We think that the obtained results might improve the therapy with cladribine and also reduce the risk of the adverse effects and damage of the tissues. Moreover, knowledge of how cladribine influences lung morphology might improve the design of therapeutic strategies.

References
