Antibody-targeted horseradish peroxidase associated with indole-3-acetic acid induces apoptosis in vitro in hematological malignancies

Leandro F.F. Dalmazzo a, Bárbara A. Santana-Lemos a, Rafael H. Jácomo a, Aglair B. Garcia a, Eduardo M. Rego a, Luiz M. da Fonseca b, Roberto P. Falcão a,∗

a Department of Internal Medicine, Hematology Division, and Center for Cell-based Therapy, Medical School of Ribeirão Preto, University of São Paulo, Av. Bandeirantes, 3900, 14048-900 Ribeirão Preto, SP, Brazil
b Department of Clinical Analysis, School of Pharmaceutical Sciences, São Paulo State University, Araraquara, SP, Brazil

1. Introduction

One of the goals of antitumor therapy is to selectively target toxic agents to tumor cells, sparing normal tissues of injury, which can be achieved by antibodies that recognize specific markers on the tumor cell surface. In this regard, enzymes capable of activating prodrugs to active drugs have been the focus of considerable recent interest, and conjugation of the enzyme to an antibody could allow it to be targeted selectively to the tumor after intravenous administration, a procedure called antibody-directed enzyme prodrug therapy (ADEPT) [1].

Indole-3-acetic acid (IAA), when oxidized by horseradish peroxidase (HRP), is transformed into cytotoxic molecules capable of inducing cell injury. The aim of this study was to test if, by targeting hematopoietic tumors with HRP-conjugated antibodies in association with IAA treatment, there is induction of apoptosis. We used two lineages of hematologic tumors: NB4, derived from acute promyelocytic leukemia (APL) and Granta-519 from mantle cell lymphoma (MCL). We also tested cells from 12 patients with acute myeloid leukemia (AML) and from 10 patients with chronic lymphocytic leukemia (CLL). HRP targeting was performed with anti-CD33 or anti-CD19 antibodies (depending on the origin of the cell), followed by incubation with goat anti-mouse antibody conjugated with HRP. Eight experimental groups were analyzed: control, HRP targeted, HRP targeted and incubated with 1, 5 and 10 mM IAA, and cells not HRP targeted but incubated with 1, 5 and 10 mM IAA. Apoptosis was analyzed by flow cytometry using annexin V-FITC and propidium iodide labeling. Results showed that apoptosis was dependent on the dose of IAA utilized, the duration of exposure to the prodrug and the origin of the neoplasia. Targeting HRP with antibodies was efficient in activating IAA and inducing apoptosis.

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Our aim was to evaluate apoptosis induced by IAA/HRP in myeloid and lymphoid malignancies using cell lineages and primary tumor cells from patients with acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL).

2. Material and methods

2.1. Cell lineages

We used the cell lineages NB4 and Granta-519. The first is derived from blasts of human acute promyelocytic leukemia (APL), harbors the t(15;17)(q22.q12) translocation and expresses typical immunophenotypic markers such as CD33, CD13, CD15 and cytoplasmic myeloperoxidase [18]. The Granta-519 cell lineage is derived from human mantle cell lymphoma (MCL), harboring the t(11;14)(q13;q32) translocation with overexpression of cyclin D1 [19]. These cells also express characteristic surface markers such as CD19, FMC7, CD23 and lambda chain [19]. The NB4 lineage was kindly provided by Professor Pier Paolo Pandolfi (Harvard Medical School, Boston, USA) and Granta-519 by Professor Belinda Pinto Simões (GSF-Hämatologikum, Munich, Germany). Primary human microvascular endothelial cells from lung (HMVEC-L) (Lonza, Walkersville, MD, USA) were co-cultured with Granta-519 to evaluate the effects of IAA/HRP on normal cells.

2.2. Patients

From January/2007 to December/2008, 22 patients from the University Hospital of the Medical School of Ribeirão Preto were enrolled in this study after giving written informed consent. Bone marrow was aspirated from 12 patients with AML (six with APL and six with AML other than APL) and peripheral blood was obtained from 10 patients with CLL and mononuclear cells were isolated by Ficoll Hypaque density gradient centrifugation (Sigma–Aldrich, St. Louis, MO, USA). The patients were diagnosed according to classical criteria established by the World Health Organization. The clinical and laboratory records of these patients were reviewed. Approval from the local Ethics Committee was obtained (protocol number 4342/2006).

2.3. HRP targeting

The HRP targeting to the tumor cells was performed based on indirect labeling utilizing monoclonal antibodies. First, 0.5 × 10⁶ cells were incubated with 5 μL of murine anti-CD33 (NB4 and AML primary cells) or anti-CD19 (Granta-519 or CLL primary cells). Both antibodies were purchased from BD Pharmingen (San Diego, USA) and 0.5 μg/mL propidium iodide (PI). Flow cytometric analysis was performed on a FACScalibur (Becton Dickinson, San Jose, CA, USA), where 10,000 events were acquired for fluorescence measurements. Annexin V-positive cells were considered to be apoptotic.

2.4. Cell culture

Eight experimental groups were analyzed: controls (in which no antibody was added), cells targeted with HRP, cells targeted with HRP and incubated with 1, 5 or 10 mM IAA (Vetec Quimica Fina Ltd., Rio de Janeiro, Brazil). NB4 and Granta-519 cells were cultured at 3% CO₂/37 °C in quintuplicate and tested for apoptosis after 2, 8, 18, 24 and 48 h of incubation. Similarly, cells from AML and CLL patients were divided into 16 aliquots and cultured under the same conditions for 2 and 24 h. IAA was dissolved in 10% ethanol, and controls were treated with the same vehicle. The final ethanol concentration in each well was 1%.

Separately, HMVEC-L cells were grown in ECM-2MV medium (Lonza, Walkersville, MD, USA) supplemented as indicated by the manufacturer. A total of 0.5 × 10⁶ Granta-519 cells were added to this culture after HMVEC-L cells reached 90% confluence (around 0.5 × 10⁶ cells). Six experimental groups were incubated for 24 h and then analyzed: controls (in which no antibody was added), HMVEC-L cells with HRP-targeted Granta-519 cells, HMVEC-L cells with HRP-targeted Granta-519 cells treated with 1 or 5 mM IAA, HMVEC-L cells with Granta-519 cells (not HRP targeted) treated with 1 or 5 mM IAA. The HRP targeting to Granta-519 cells was performed before they were added to HMVEC-L cells culture. After 24 h, the two lineages were separated: suspended Granta-519 cells were removed and adhered HMVEC-L cells were released after 10 min incubation with trypsinization reagent (Lonza, Walkersville, MD, USA). Trypsin was then neutralized with 4 mL of Trypsin Neutralizing Solution (Lonza, Walkersville, MD, USA). In this experiment, HMVEC-L cells were exposed to the IAA/HRP effects in the culture medium. Granta-519 but not HMVEC-L cells were HRP-targeted, and the effects of this tumor treatment were analyzed in the endothelial cells by evaluation of apoptosis as described below.

2.5. Apoptosis analysis

After culture, cells were washed in binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl₂) and incubated with FITC-conjugated annexin V (BD Pharmingen, San Diego, USA) and 0.5 μg/mL propidium iodide (PI). Flow cytometric analysis was performed on a FACScalibur (Becton Dickinson, San Jose, CA, USA), where 10,000 cells were acquired for fluorescence measurements. Annexin V-positive cells were considered to be apoptotic.

2.6. Statistical analysis

A linear mixed effect model was proposed to compare differences in percentages of apoptotic cells between groups. These models are based on the assumption that their residues have normal distribution with 0 mean and \( \sigma^2 \) variance. The model proposed for each variable is as follows: 

\[ y_{ijk} = \eta_{i} + \alpha_{j} + \beta_{k} + \epsilon_{ijk} \]

where \( y_{ijk} \) is the observation of the response variable for the \( i \)th individual in the \( j \)th group and at the \( k \)th time; \( \epsilon_{ijk} \) is a random error (an intercept); \( \alpha_{j} \) is a random effect (\( i = 1, \ldots , 5; j = 1, \ldots , 8; k = 1, \ldots , 5 \)) of the \( j \)th group, \( \beta_{k} \) is the effect of the \( k \)th time, \( \delta_{jk} \) is the effect of group-by-time interaction, and \( \epsilon_{ijk} \) is the associated error with \( N(0, \sigma^2) \) distribution. These statistical analyses were performed using the SAS software version 9.

Dose–effect relations were calculated using values of four IAA doses (0, 1, 5 and 10 mM). CalcuSyn version 2.0 was the software used to calculate the \( r \) coefficient of the correlation between dose and effect (percentage of apoptotic cells) and the dose at which 50% of the effect was observed (apoptotic cells) (ED50). An \( \alpha \) value ≤ 0.05 was considered to be significant.

3. Results

3.1. Patient cohort

Tables 1 and 2 summarize the clinical characteristics of the patients, and Tables 3 and 4, the immunophenotypic characteristics. AML patients (mean age: 43 years) had mean hemoglobin level of 8.7 g/dL, a mean white blood cell count of 34,300 × 10⁶/L, and a mean platelet count of 45,000 × 10⁶/L. Of 12 patients with AML, 6 were diagnosed as having APL. Ten patients diagnosed with CLL were enrolled. The mean age of these patients was 69 years, mean hemoglobin level was 13.2 g/dL, mean white blood cell count 60,700 × 10⁶/L, mean lymphocyte count 48,660 × 10⁶/L, and mean white blood cell count 32,600 × 10⁶/L.
Table 2
Clinical characteristics from CLL patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Hemoglobin (g/dL)</th>
<th>WBC (×10⁶/L)</th>
<th>Lymphocytes (×10⁶/L)</th>
<th>Platelets (×10⁶/L)</th>
<th>Binet</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>62</td>
<td>M</td>
<td>14.8</td>
<td>38,600</td>
<td>31,800</td>
<td>110,000</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>85</td>
<td>M</td>
<td>12.3</td>
<td>35,500</td>
<td>25,200</td>
<td>210,000</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>M</td>
<td>10.5</td>
<td>63,100</td>
<td>41,200</td>
<td>217,000</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>M</td>
<td>14.6</td>
<td>30,600</td>
<td>25,800</td>
<td>319,000</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>66</td>
<td>F</td>
<td>13.3</td>
<td>55,300</td>
<td>43,700</td>
<td>196,000</td>
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<tr>
<td>6</td>
<td>72</td>
<td>M</td>
<td>13.9</td>
<td>76,300</td>
<td>45,800</td>
<td>413,000</td>
<td>B</td>
</tr>
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<td>7</td>
<td>77</td>
<td>M</td>
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<td>B</td>
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<td>9</td>
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<td>M</td>
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<td>217,000</td>
<td>A</td>
</tr>
<tr>
<td>10</td>
<td>49</td>
<td>F</td>
<td>13.3</td>
<td>26,300</td>
<td>18,100</td>
<td>162,000</td>
<td>A</td>
</tr>
</tbody>
</table>

WBC: white blood cells.

Table 3
Immunophenotypic characteristics from AML patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Immunophenotypic markers</th>
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<tbody>
<tr>
<td></td>
<td>Positive markers</td>
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<tr>
<td>1</td>
<td>CD33, CD13, HLA-DR, CD117, CD34, CD11b, MPO</td>
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<td>2</td>
<td>CD33, CD13, HLA-DR, CD117, CD34, MPO</td>
</tr>
<tr>
<td>3</td>
<td>CD33, CD13, HLA-DR, CD117, CD34, MPO</td>
</tr>
<tr>
<td>4</td>
<td>CD33, CD13, HLA-DR, CD117, CD34, CD15, MPO</td>
</tr>
<tr>
<td>5</td>
<td>CD33, CD13, HLA-DR, CD117, CD34, MPO</td>
</tr>
<tr>
<td>6</td>
<td>CD33, CD13, HLA-DR, CD117, CD11b, MPO</td>
</tr>
<tr>
<td>7</td>
<td>CD33, CD13, CD117, MPO</td>
</tr>
<tr>
<td>8</td>
<td>CD33, CD13, CD117, CD15, MPO</td>
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<tr>
<td>9</td>
<td>CD33, CD13, CD117, MPO</td>
</tr>
<tr>
<td>10</td>
<td>CD33, CD13, CD117, MPO</td>
</tr>
<tr>
<td>11</td>
<td>CD33, CD13, CD117, MPO</td>
</tr>
<tr>
<td>12</td>
<td>CD33, CD13, CD117, MPO</td>
</tr>
</tbody>
</table>

Positive reaction to a given antibody is defined as a minimum threshold of 20% positive blasts to the respective antigen. MPO: myeloperoxidase.

platelet count 207,300 × 10⁶/L. All patients presented at the initial stages of the disease (Binet classification A or B).

3.2. Apoptosis in NB4 lineage

Fig. 1A shows the percentage of apoptotic cells in NB4 samples treated with IAA associated or not with antibody-directed HRP. All the control groups (cells only, cells targeted with HRP and not incubated with IAA and cells incubated with IAA and not targeted with HRP) presented low percentages of apoptotic cells with no statistical differences between them. After 8 h of incubation, only cells targeted with HRP and treated with 10 mM IAA presented a significantly higher percentage of apoptotic cells compared to control groups (mean 32.2% versus 11.1–14.9% in the control groups, \( p \leq 0.05 \)). After 18 h, cells targeted and treated with 5 and 10 mM IAA presented 52.3 and 32.2% rates of apoptosis, respectively (controls ranged from 10.2 to 15.7%, \( p \leq 0.05 \)). After 24 and 48 h, all groups targeted with HRP and treated with 1, 5 and 10 mM IAA presented a significantly higher percentage of apoptotic cells compared to the control groups (\( p \leq 0.05 \)).

3.3. Apoptosis in the Granta-519 lineage

Apoptosis induction by the different treatments in Granta-519 cells is shown in Fig. 1B. We found higher apoptosis in all cells targeted and treated with 1, 5 and 10 mM IAA starting at 8 h of culture compared to the control groups. After 8 h, cells targeted and treated with 1, 5 and 10 mM IAA presented 25.2, 31.8 and 41.3% rates apoptotic cells, respectively, while in the control groups these percentages ranged from 13.1 to 15.6% (\( p \leq 0.05 \)). After 18, 24 and 48 h, apoptosis in targeted and treated cells reached levels of approximately 70%, while control groups presented levels between 8.9 and 20.6% (\( p \leq 0.05 \)).

3.4. Apoptosis in the HMVEC-L lineage

The percentages of apoptotic cells in the HMVEC-L lineage that was co-cultured with Granta-519 are shown in Fig. 2. After 24 h of
incubation, all groups presented similar percentages of apoptosis ranging from 4.8 to 8.2%, with no significant differences.

3.5. Apoptosis in CLL patients

The mean percentages of apoptotic cells detected in CLL patients’ samples are shown in Fig. 3A. After 2 h of incubation, all samples presented similar percentages of apoptosis ranging from 22.8 to 28.9%, with no statistical difference. Analysis at 24 h demonstrated that the groups targeted and treated with 1, 5 and 10 mM IAA had higher percentages of apoptotic cells (58.5, 81.3 and 93.3%, respectively) compared to the control groups (35.7–53.7%, \( p \leq 0.05 \)) (Fig. 3A).

3.6. Apoptosis in AML patients

3.6.1. Non-APL patients

In AML other than APL, the percentage of apoptotic cells after 2 h of incubation was similar in all groups, ranging from 8.9 to 15.5%,
Fig. 3. Percentage of apoptotic cells according to the group of treatment and time of incubation (2 and 24 h). (A) CLL patients’ results, (B) AML patients’ results and (C) APL patients’ results. The bars indicate mean ± SE. Statistical analysis is pointed according to the level of significance (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001), comparing the treated group to the control group.

with no statistical differences. In 24 h samples, targeted cells which were also treated with 1, 5 and 10 mM IAA presented higher percentages of apoptosis (44.4, 64.7 and 78.8% respectively, while in the control groups apoptosis ranged from 18.5 to 27.8%, p ≤ 0.05) (Fig. 3B).

3.6.2. APL patients

In APL patients’ samples treated for 2 h, the percentage of apoptotic cells was similar in all groups, ranging from 7.7 to 12.1%, with no statistical differences. Twenty-four hours analysis demonstrated a higher percentage of apoptotic cells only in the targeted groups treated with 5 and 10 mM IAA (51.9 and 75.9%, respectively, p ≤ 0.05). In the control groups and in the group targeted with HRP and treated with 1 mM IAA no differences were noted (the values ranged from 18.1 to 44.8%). In these experiments, we detected that samples not targeted with HRP but treated with 10 mM IAA showed higher apoptosis induction compared to other controls (44.8%, while the other controls ranged from 18.1 to 26.8%, p ≤ 0.05) (Fig. 3C).

3.7. Dose–effect correlation

The ED50 (dose at which 50% of the cells were apoptotic) and the r coefficient of the correlation between dose and effect are described in Table 5 for tumor cells treated for 24 h. By comparing the Granta-519 lineage with NB4, we could demonstrate that the ED50 for the MCL lineage was about 1000 times lower than the values calculated for the NB4 lineage. Cells from CLL patients’ samples also presented a much lower ED50 than that of cells from AML and APL samples. Besides, the r coefficient demonstrated a good dose–effect correlation in all groups, with few exceptions.

Table 5

<table>
<thead>
<tr>
<th>Group</th>
<th>ED50 (mM)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB4</td>
<td>112.7</td>
<td>0.90</td>
</tr>
<tr>
<td>Granta-519</td>
<td>0.11</td>
<td>0.95</td>
</tr>
<tr>
<td>AML patients</td>
<td>0.19</td>
<td>0.92</td>
</tr>
<tr>
<td>APL patients</td>
<td>2.5</td>
<td>0.88</td>
</tr>
<tr>
<td>CLL patients</td>
<td>0.003</td>
<td>0.84</td>
</tr>
</tbody>
</table>

p ≤ 0.05). In the control groups and in the group targeted with HRP and treated with 1 mM IAA no differences were noted (the values ranged from 18.1 to 44.8%). In these experiments, we detected that samples not targeted with HRP but treated with 10 mM IAA showed higher apoptosis induction compared to other controls (44.8%, while the other controls ranged from 18.1 to 26.8%, p ≤ 0.05) (Fig. 3C).

4. Discussion

Potential application of the IAA/HRP association to cancer therapy is well recognized, since it is quite evident that it can cause cell death, although toxicity mechanisms are not fully understood. The sensitivity of different cell types might not be the same[13]. Targeting the HRP enzyme to the tumor cell has already been successfully performed in vitro using gene therapy[11,12]. The first report of using antibodies in cancer therapy was published in 1980 [20], and, since then, many monoclonal antibodies have been developed and used successfully in the clinical treatment of hematopoietic patients[21–24]. Our results demonstrated that the antibody-directed enzyme/prodrug therapy (ADEPT) IAA/HRP could induce apoptosis in all the cells tested and, therefore, may enhance the anti-leukemia effect of monoclonal antibodies. The toxicity to Granta-519 lineage and CLL cells, both tumors of lymphoid origin, was higher compared with NB4 lineage. AML and APL cells, as demonstrated by the much higher baseline rate of apoptosis, which in turn impacts on their lower ED50 values, especially in CLL patients’ samples.

The IAA doses used appear to be much higher than those previously used in vitro [2,3,5,11]. Probably, the HRP targeting using antibodies required these higher doses to achieve the expected toxic effects. Whether this will be possible in vivo remains to be evaluated.

To evaluate the fate of non-targeted normal cells, the effects of IAA/HRP on the HMVEC-L endothelial lineage was analyzed when these cells were co-cultured with HRP-targeted Granta-519 cells. The endothelial cells suffered minimal deaths, and the percentage of apoptosis was not different between them, even in the groups that were co-cultured with HRP-targeted Granta-519 cells. The endothelial cells suffered minimal deaths, and the percentage of apoptosis was not different between them, even in the groups that were co-cultured with HRP-targeted Granta-519 cells. The toxicity to Granta-519 lineage and CLL cells, both tumors of lymphoid origin, was higher compared with NB4 lineage. AML and APL cells, as demonstrated by the much higher baseline rate of apoptosis, which in turn impacts on their lower ED50 values, especially in CLL patients’ samples.

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target HRP to more specific tumor markers, sparing normal cells from injury, a fact that remains to be determined.

In APL patients, the group of cells that was not HRP targeted but was treated with 10 mM IAA presented higher apoptosis when compared to the other control groups, a fact suggesting that HRP is more effective in activating IAA than myeloperoxidase.

In summary, the association of HRP and IAA can cause apoptosis in human hematopoietic tumors which was dependent on the dose of IAA used, on the duration of exposure to the prodrug and on the origin of the neoplasia. The targeting of HRP using antibodies was effective for this purpose. To consider clinical applications, the mechanisms of toxicity must be better elucidated, the effects of IAA in humans must be carefully studied and the impact of the observed apoptosis in tumor therapy and in normal cells must be better assessed in in vivo models.

Conflict of interest

The authors declare that they have no potential conflicts of interest.

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Contribution: LFFD provided the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article, revised it critically for important intellectual content, and approved the final version to be submitted; BASL analyzed data, drafting of manuscript and was responsible for important intellectual content; RHJ contributed with statistical analysis; ABG performed the flow cytometry analysis; EMR and LMF analyzed data, drafting of manuscript and were responsible for important intellectual content; RPF revised the article critically for important intellectual content and gave final approval of the version to be submitted.

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