Promising Trial for Treatment of Chronic Myelogenous Leukemia Using Plasma Technology

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ABSTRACT: This article presents a trial of the treatment of chronic-phase (CP) and accelerated-phase (AP) chronic myelogenous leukemia (CML) that is resistant or only partially responded to chemotherapy. Blood samples of 6 cases diagnosed with CML were studied and compared with a control group. The first 3 cases were AP CML resistant to imatinib and nilotinib. The other 3 partially responded to the chemotherapy and returned to CP CML. Triple blood cultures for each case were exposed to a cold, pulsed, atmospheric pressure plasma jet for different durations (40, 80, and 120 seconds). Hematological, cytogenetic, and biochemical investigations were done before and after plasma jet exposure. The results showed an increase in necrotic and apoptotic cell counts and a decrease in the number of characteristic nucleoplasmic bridges (multinucleated threadlike shape). Concentrations of transforming growth factor-β1 and arginase decreased in the CML blood samples after exposure to plasma jet. This type of nonthermal plasma can kill cancer cells and prevent the cells from dividing, especially for the 80-second duration.

KEY WORDS: plasma jet, cytokinesis block micronucleus assay, chronic myelogenous leukemia, TGF-β1, arginase

I. INTRODUCTION

Cold pulsed atmospheric pressure plasma is a multicomponent system that includes such biological active agents as charged particles, reactive nitrogen and oxygen species, and electromagnetic radiations (ultraviolet, visible, infrared light). Therapeutic application of plasma at or in the human body is a challenge both for medicine and plasma physics. The main characteristic of plasma sources for use in medical therapy is its direct application on or in the human body. Therefore, plasma effects on mammalian cells are of basic interest. In vitro tests to characterize plasma–cell interactions should include basic cellular parameters such as morphology, viability, or proliferation and the cellular responses life influence on DNA or cellular proteins. Moreover, plasma acts at the cellular level to remove diseased tissue without inflammation and damage, to suppress infections, and to modulate the viability (apoptosis/necrosis) of tumoral cells.
Chronic myelogenous leukemia (CML) is a prototypical stem cell malignancy with a natural course of progression from an initial chronic phase (CP) to an accelerated phase (AP) and blast crisis (BC). Progression to BC is associated with acquisition of additional chromosomal aberrations beyond the underlying t(9:22) chromosomal translocation that characterizes CML.\(^5\)

Nilotinib is pharmacologically related to imatinib mesylate and dasatinib; these drugs are inhibitors of Bcr-Abl tyrosine kinase. Imatinib resistance can be defined as lack of complete hematologic response in patients with CP CML or as a failure to return to CP in patients with CML in AP or BC. The majority of patients with imatinib-resistant CML either have a secondary Bcr-Abl mutation that impairs the ability of the kinase to adopt the closed conformation to which imatinib binds or that directly interferes with drug binding. Drug resistance is associated with reactivation of Bcr-Abl signal transduction.\(^6,7\)

In CML, the increase in mature granulocyte counts (small percentage because of dilution of the differential count) results in a total white blood cell count of 20,000–60,000 cells/µL. A mild increase in basophiles and eosinophils is present and becomes more prominent during the transition to acute leukemia. These mature neutrophils, or granulocytes, have decreased apoptosis, resulting in the accumulation of long-lived cells with few or absent enzymes, such as alkaline phosphatase.\(^8\)

The most striking abnormality is found in the white blood cell series. Cells at all stages of granulopoiesis (including early progenitor cells) are found in the peripheral blood to the extent that it can resemble bone marrow aspirate. Basophil and eosinophil counts are increased.\(^9\)

The cytokinesis-block micronucleus (CBMN) cytome assay is a comprehensive system for measuring DNA damage, cytostasis, and cytotoxicity. DNA damage events are scored specifically in once-divided binucleated cells and include micronuclei (MNi), a biomarker of chromosome breakage and/or whole chromosome loss; nucleoplasmic bridges (NPBs), a biomarker of DNA misrepair and/or telomere end-fusions; and nuclear buds, a biomarker of elimination of amplified DNA and/or DNA repair complexes. Cytostatic effects are measured via the proportion of mono-, bi-, and multinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ratios.\(^10\)

Transforming growth factor (TGF)-β1 signaling regulates several different biological processes involving cell growth, differentiation, apoptosis, motility, angiogenesis, epithelial-to-mesenchymal transition, and extracellular matrix production that affect embryonic development and the pathogenesis of various diseases including cancer; its effects depend on the cellular context and physiological environment. Despite its inhibitory effect on growth, in certain conditions TGF-β1 may act as a promoter of cell proliferation and invasion.\(^11\) In addition, TGF-β1 signaling through its unique transmembrane receptor serine–threonine kinase plays a complex role in carcinogenesis, having both tumor suppressor and oncogenic activities. Tumor cells often escape from the antiproliferative effects of TGF-β1 by mutational inactivation or dysregulated expression of components in its signaling pathway.\(^12\)

Arginase is one of the essential enzymes in the terminal stages of the urea cycle in
the liver, which participates in the elimination of ammonia from the human body. In addition to liver tissue, arginase is also present in many other human tissues and in circulating blood cells, especially erythrocytes and leukocytes. Arginase activity is higher in cancerous tissue than in their normal counterparts, and this might be consistent with the role of arginase in ornithine biosynthesis. Therefore, the pattern of enzymatic alterations may be linked with the malignant state and the progression of cancerous cells in the tumor. Differences in activities or concentrations of certain enzymes between cancer cells and their normal counterparts might be useful as biological markers of malignancy and/or aggressiveness in particular tumors.

This study presents a trial of treatment for patients with CML, especially those who were resistant to or partially treated with chemotherapy.

II. MATERIALS AND METHODS

A. Characteristics of the Plasma Jet

The plasma generator used in these experiments consisted of a negative direct current source, a Blumlein-type pulse-forming network (E-PFN), and a dynamic spark gap switch. A triggered spark gap switch is used as a closing switch for the E-PFN. The E-PFN has 4 stages of the LC ladder, composed of 5 nF of capacitor and 3 μH of inductor. The characteristic impedance (2√L/C) and the pulse width (2N√LC) of E-PFN, calculated from the capacitance (C) and inductance (L) of the LC ladder and the number (N) of LC ladder stages, are approximately 49 Ω and 1.0 μsec, respectively. The configuration of this apparatus is as follows: The charging resistance is 50 kΩ; this value corresponds to a charging RC time constant of 1 msec, which is 40 times faster than the repetition rate of the pulse. A schematic of the pulsed atmospheric pressure plasma jet (APPJ) for generating high-voltage (HV) pulsed, cold atmospheric plasma jets is shown in Fig. 1. The HV wire electrode, which is made of copper wire, is inserted into the hollow barrel of a syringe. The distance between the tip of the HV electrode and the nozzle is 0.5 cm.

HV pulsed direct current voltage (amplitudes up to 25 kV, repetition rate up to 25 Hz) is applied to the HV electrode and helium gas is injected into the hollow barrel. This device uses a medical syringe (made of an insulating material cylinder). The gas is fed into the system via a flow meter. The applied voltage to and the discharge current through the discharge chamber are measured using a homemade voltage divider connected between the 2 electrodes and a current monitor, which is located on the return to the ground. The signals from the voltage divider and the current monitor are recorded using a digitizing oscilloscope (Lecroy) with a 200-MHz bandwidth.

The HV pulses are applied between the needle electrode positioned inside a dielectric cylinder (a simple medical syringe), and a metal ring is placed on the exterior of this cylinder. To obtain electric discharges at atmospheric pressure, HV pulses (tens of kilovolts), which have limited duration (hundreds of nanoseconds) and are repeated (tens of
pulses per second), in addition to an inert gas (argon), are introduced into the cylinder. The gas flows are in the range of 0.5–10 L/min. The discharge takes place between the top of the metallic needle and a metallic ring fitted on the outer surface of the syringe. Under optimal conditions, plasma is emitted as centimeter-long jets, just millimeters in diameter or even smaller.

The working gases are supplied by high-pressure cylinders. Gas pressure regulators are used to reduce the pressure of gases to a workable level. Then, gas flow controllers deliver the gases with the desired flow. For voltage amplitudes of 15–18 kV, the plasma jet is very weak. The plasma jet disappears at voltage amplitudes lower than 15 kV. When argon is injected from the gas inlet and an HV pulse (26 kV) is applied to the electrode, the plasma jet is generated, and a plasma plume reaching a length of 21 mm is launched through the end of the tube and into the surrounding air. The length of the plasma plume is adjusted using the gas flow rate and the applied voltage.

B. Chemicals

The chemicals for use in the blood culture were purchased from GIBCO-BRL; heat-inactivated fetal calf serum from Sigma-Aldrich Chemical Co. (St. Louis, MO); TGF-β1 enzyme-linked immunosorbant assay kit from DRG International, Inc.; and arginase from BioDiagnostics, Inc. (River Falls, WI).

C. Blood Sampling

Blood samples obtained from 6 volunteer patients with CML, the first 3 of whom were in AP CML and were imatinib resistant (treatment-resistant [TR] cases); the others were
in CP CML and partially responded to the imatinib treatment (treatment [T] cases). The diagnosis was based on clinical examination and laboratory evaluation, which were carried out by the consulting medical staff. Further, healthy subjects (control group) also were investigated and had no history or signs of leukemia. The control group and the patient groups were matched for ethnic background, sex, smoking, and age.

All subjects were gave informed consent for participation in this study. The donors were selected according to current International Programme on Chemical Safety guidelines for the monitoring of genotoxic effects of carcinogens in humans.16 Venous blood samples were collected under sterile conditions in a heparinized vacutainer tube (5-mL volume; Becton Dickinson) containing lithium heparin as the anticoagulant.

### D. Experimental Design

The heparinized blood from each volunteer was divided into 4 groups for each case (TR and T cases). One was unexposed and the other 3 were applied directly to the APPJ at a distance of 3 cm from the blood surface. In each group, 3 time points were selected for the study (40, 80, and 120 seconds). The first 4 groups of TR cases were categorized as the following: TR, TR-D1, TR-D2, and TR-D3 groups. The other 4 groups of T cases were categorized as the following: T, T-D1, T-D2, and T-D3 groups. All of these groups were matched with a group of healthy subjects, which represent the negative control (C group).

After exposure, the blood was incubated at 37°C for 20 hours before initiating cultures and biochemical investigations. Blood films for each group were done before and after exposure to the APPJ.

1. **Blood Film Preparation**

Blood films were prepared using a typical protocol according to the methodology described by Berend.17

2. **Blood Culture**

Triple blood cultures were set up for 72 hours for each sample according to the protocol described by Evans and O’Riodran18 and its modification by Fenech10.

3. **Cytokinesis-Block Micronucleus Assay**

CBMN assay, performed as described by Fenech,10,19 is referred to as the cytome assay. We recorded mono-, bi-, tri-, and quadrinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ratio. Moreover, the frequency of MNi and NPBs in the cells were
detected for 1000 cells in each sample.

4. Determination of Arginase

The method used by BioDiagnostics for plasma arginase concentration was based on the colorimetric determination of urea by condensation with diacetylmonoxime in an acid medium in the presence of ferric chloride (oxidant) and carbazide (accelerator).20

5. Determination of TGF-β1

TGF-β1 was determined based on the sandwich principle using a TGF-β1 enzyme-linked immunosorbant assay kit (DRG International, Inc.), according to the manufacturer’s instructions.

6. Statistical Analysis

Data were presented as a distribution analysis and as percentages and means ± standard errors; they were analyzed using the 2-way analysis of variance F test, as described by Abramowitz and Stegum.21 The level for statistical significance was $P < 0.05$.

III. RESULTS

Table 1 shows that the TR group had a low incidence of mature cells (56.33%). Characteristic percentages of cell types in the patients with CML were scored as follows: basophiles and eosinophils, 21%; lymphocytes, 12.7%; and neutrophils, 18.7%. Immature cells were represented as a percentage (40.3%). Low frequencies of necrotic cells were detected in this group. All data from the TR group were significant difference when compared with the C group (Fig. 2).

After exposure to APPJ, the parameters of the TR-D1, TR-D2, and TR-D3 groups changed as follows: decreases in the percentages of the mature cells (eosinophils, basophiles) and immature cells (progenitor cells and myeloblasts) and increases in the ratio of the necrotic cells.

The results point to statistical differences between the means of the percentages of necrotic and immature cells between the TR group and the exposed TR-D1, TR-D2, and TR-D3 groups. The TR-D2 group scored a significant improvement in the frequencies of basophils and eosinophils compared with the TR, TR-D1, and TR-D3 groups (Fig. 3).

Table 2 shows that the T group had a significant decrease in mature cells when compared with the C group and a nonsignificant increase in necrotic cell frequencies (Fig. 4). The statistical differences between the means of the percentages of progenitor cells

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TABLE 1: Differential counts of mature and immature cells of peripheral blood of exposed and non-exposed TR- and control groups (count/100 cells)

<table>
<thead>
<tr>
<th>Group</th>
<th>C-group</th>
<th>TR-group</th>
<th>TR-D1 group</th>
<th>TR-D2 group</th>
<th>TR-D3 group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature cells</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
</tr>
<tr>
<td>Basophiles</td>
<td>0.33±0.3</td>
<td>1.00±9</td>
<td>0.88±6.3</td>
<td>0.33±2.3</td>
<td>0.58±6.6</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.58±6</td>
<td>0.58±12</td>
<td>0.33±9.3</td>
<td>0.67±6.7</td>
<td>0.88±5.7</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.58±35</td>
<td>1.77±12.7</td>
<td>0.88±11.3</td>
<td>0.67±12.7</td>
<td>1.45±9.3</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.88±50.7</td>
<td>1.33±18.7</td>
<td>0.88±12.3</td>
<td>0.58±19</td>
<td>1.73±14</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.33±7.7</td>
<td>1.16±4</td>
<td>0.88±4.7</td>
<td>0.58±4</td>
<td>0.88±2.7</td>
</tr>
<tr>
<td>Total count of mature cells</td>
<td>0.33±99.7</td>
<td>0.67±56.33</td>
<td>2.52±44</td>
<td>2.34±44.7</td>
<td>2.67±37.7</td>
</tr>
<tr>
<td>Progenitor cells</td>
<td>0.0±0</td>
<td>1.77±16.7</td>
<td>1.16±16</td>
<td>1.45±12.3</td>
<td>0.88±11.7</td>
</tr>
<tr>
<td>Myeloblasts</td>
<td>0.0±0</td>
<td>1.67±21.7</td>
<td>0.88±15.7</td>
<td>0.58±10</td>
<td>0.88±14.3</td>
</tr>
<tr>
<td>Band cells</td>
<td>0.33±3</td>
<td>1.16±2</td>
<td>0.33±1.7</td>
<td>0.00±0</td>
<td>0.33±0.3</td>
</tr>
<tr>
<td>Total count of immature cells</td>
<td>0.27±0.3</td>
<td>0.27±40.3</td>
<td>0.72±33.3</td>
<td>1.09±22.3</td>
<td>0.98±26.3</td>
</tr>
<tr>
<td>Necrotic cells</td>
<td>0.00±0</td>
<td>0.33±3.3</td>
<td>1.86±22.7</td>
<td>1.53±33</td>
<td>1.53±36</td>
</tr>
</tbody>
</table>

* P < 0.05  
a: P-value Significant when compared with C-group. b: P-value Significant when compared with TR-group. c: P-value Significant when compared with TR-D1group. d: P-value Significant when compared with TR-D2group.

FIG. 2: Chronic myelogenous leukemia cells from the treatment-resistant group showing high incidence of immature cells and low frequency of mature cells, which include the following cell types: basophils (B), eosinophils (E), lymphocytes (L), progenitor cells (Prog), and myeloblasts (Myelo).
in the TR-D2 and TR-D3 groups, however, were significant. The exposed groups (T-D1, T-D2, and T-D3 groups) showed improvement in the ratio of the cell types compared with the C group, but the differences were not significant (Fig. 5).

Table 3 shows that the TR group had a low incidence of binucleated cells and low expression of MNi. In contrast, this group is characterized by high percentages of NPBs with a unique form (multinucleated thread-like bridges) and mononucleated cells (Fig. 6). In the C group, the frequency of the mononucleated cells was 2-fold more than that of the binucleated count (Fig. 7). On the other hand, there was a nonsignificant difference between the incidences of mononucleated and binucleated cells in the TR-D2 group compared with the C group.

The expression of MNi in the exposed groups (TR-D1, TR-D2, and TR-D3) was higher than that of the TR group, but it was only significant in the TR-D2 group. Also, this increase occurred for the expression of the necrotic and apoptotic cells, especially in the TR-D3 group (Fig. 8).

Table 4 shows that the frequency of mononucleated cells in the T group was 3-fold more than the frequency of the binucleated cells comparing with two folds only detected in the C-group (Fig. 7). The expression of the MNi in the T group was not significantly different compared with its values in the exposed groups. In addition, the results state that the shape and counts of NPBs in the T group are more diverse than those of NPBs in the TR group.

A significant increase was detected for apoptotic cell count in the T-D2 and

\textit{FIG. 3}: Chronic myelogenous leukemia cells in the treatment group showing high incidence of immature and necrotic cells and more expression of mature cells, which include the following cell types: basophils (B), eosinophils (E), lymphocytes (L), monocytes (M), neutrophils (N), progenitor cells (Prog), myeloblasts (Myelo), and necrotic cells (Necr)
TABLE 2: Differential counts of mature and immature cells of peripheral blood of exposed and non-exposed T- and control groups (count/100 cells).

<table>
<thead>
<tr>
<th>Group</th>
<th>C-group</th>
<th>T-group</th>
<th>T-D1 group</th>
<th>T-D2 group</th>
<th>T-D3 group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mature cells</strong></td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
</tr>
<tr>
<td>Basophiles</td>
<td>0.33±0.3</td>
<td>1.16±6</td>
<td>0.88±6.7</td>
<td>0.58±4</td>
<td>0.00±4</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.58±6</td>
<td>0.58±7</td>
<td>0.33±7.3</td>
<td>0.33±7.7</td>
<td>0.58±5</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.58±35</td>
<td>0.00±30</td>
<td>1.77±29.7</td>
<td>1.33±28.7</td>
<td>0.88±30.3</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.88±50.7</td>
<td>2.33±3.33</td>
<td>0.67±29.3</td>
<td>0.33±34.7</td>
<td>0.33±33.7</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.33±7.7</td>
<td>0.58±5</td>
<td>0.00±5</td>
<td>1.20±6.3</td>
<td>0.33±6.3</td>
</tr>
<tr>
<td>Total count of mature cells</td>
<td>0.33±99.7</td>
<td>1.86±81.3</td>
<td>2.00±78.0</td>
<td>0.67±81.3</td>
<td>0.33±79.3</td>
</tr>
<tr>
<td>Progenitor cells</td>
<td>0.0±0.0</td>
<td>0.33±5.7</td>
<td>0.58±6</td>
<td>0.33±4.3</td>
<td>0.33±4.7</td>
</tr>
<tr>
<td>Myeloblasts</td>
<td>0.0±0.0</td>
<td>0.88±8.7</td>
<td>1.16±8</td>
<td>0.33±7.3</td>
<td>0.0±8</td>
</tr>
<tr>
<td>Band cells</td>
<td>0.33±0.3</td>
<td>0.33±0.3</td>
<td>0.33±0.3</td>
<td>0.33±0.3</td>
<td>0.33±0.7</td>
</tr>
<tr>
<td>Total count of immature cells</td>
<td>0.27±14.7</td>
<td>0.88±14.7</td>
<td>1.45±14.3</td>
<td>0.58±12</td>
<td>0.33±13.3</td>
</tr>
<tr>
<td>Necrotic cells</td>
<td>0.00±0.0</td>
<td>2.08±4.0</td>
<td>1.20±7.7</td>
<td>0.33±6.7</td>
<td>0.33±7.3</td>
</tr>
</tbody>
</table>

*P < 0.05
B: P-value Significant when compared with T-group. C: P-value Significant when compared with T-D1 group. D: P-value Significant when compared with T-D2 group.

T-D3 groups compared with its incidence in the T group. Most data presented for the T-D2 and T-D3 groups were significantly different when compared with the C, T, and T-D1 groups—especially the incidences of tri- and quadrinucleated cells (Fig. 10).

Tables 5 and 6 display the results of the biochemical investigations of exposed and unexposed TR and T groups in comparison with the C group. Table 5 records that the arginase concentrations was higher in the TR group than the C group by 3.98-fold. On the other hand, the TGF-β1 value in the TR group was higher than its value in the C group (4.1-fold). There was a gradually significant decrease in the concentrations of arginase and TGF-β1 after exposure in the TR-D1 to TR-D2 groups, but the TR-D3 group recorded increases in both arginase and TGF-β1 concentrations higher than those of the TR group.

Table 6 shows that the arginase concentration was higher in the T group than in the C group by 2.33-fold. In addition, the TGF-β1 concentration in the T group was higher than that in the C group (by 3.9-fold). The doses affected the concentrations of arginase and TGF-β1, gradually decreasing from D1 to D3; however, an insignificant increase in only arginase was detected at D2 when compared with D1.
IV. DISCUSSION

Imatinib mesylate, a Bcr-Abl kinase inhibitor has been very successful in the treatment of CML. However, the majority of patients achieving cytogenetic remissions with imatinib treatment have molecular evidence of persistent disease, and residual Bcr-Abl progenitor can be detected.\(^{22}\)

Imatinib has replaced both allogenic stem cell transplantation and interferon-\(\alpha\) as first-line treatment for CP CML.\(^{22}\) Imatinib is a first-generation tyrosine kinase inhibitor of C-ABL oncogene I receptor tyrosine kinase (ABL\(I\)), arginase, and platelet-derived growth factor.\(^{23}\) Imatinib targets the adenine triphosphate binding site within the breakpoint cluster region (BCR)-ABL\(I\) fusion protein, which is the disease-causing mutant kinase in CML,\(^{24}\) and disrupts the oncogenic signal by forcing an enzymatically inactive conformation.\(^{25}\)

Imatinib resistance can be defined as the lack of a complete hematologic response in patients with CP CML or as a failure to return to CP in patients with CML in AP or BC. The majority of patients with imatinib-resistant CML have secondary Bcr-Abl mutations that either impair the ability of the kinase to adopt the closed conformation to which imatinib binds or directly interfere with drug binding. Drug resistance is asso-

**FIG. 4:** Chronic myelogenous leukemia cells in the treatment-resistant group exposed to plasma showing a decreased number of immature and progenitor cells, including high incidences of necrotic (Necr) and destructed (D) cells.
TABLE 3: The incidence of mono-, bi-, tri-, quadrinucleated, apoptic, necrotic cells, and the frequencies of micronuclei and the nucleoplasmic bridges in exposed and non-exposed TR- and control groups (counts in 1000 cells)

<table>
<thead>
<tr>
<th>Groups</th>
<th>C- group</th>
<th>TR- group</th>
<th>TR-D1 group</th>
<th>TR-D2 group</th>
<th>TR-D3 group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono. cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>X±S.E</td>
<td>%</td>
<td>X±S.E</td>
<td>%</td>
</tr>
<tr>
<td>Mono. cells+ 1 Mn</td>
<td>64.2</td>
<td>641.7 ± 18.44</td>
<td>77.7</td>
<td>776.6 ± 11.85</td>
<td>72.7</td>
</tr>
<tr>
<td>Mono. cells+ 2 Mn</td>
<td>0.5</td>
<td>4.5 ± 2.36</td>
<td>0.9</td>
<td>9.5 ± 3.49</td>
<td>0.9</td>
</tr>
<tr>
<td>BN</td>
<td>0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>BN + 1 Mn</td>
<td>0.7</td>
<td>6.7 ± 0.32</td>
<td>0.6</td>
<td>6.03 ± 1.62</td>
<td>1.1</td>
</tr>
<tr>
<td>BN + 2 Mn</td>
<td>0.0</td>
<td>0.0 ± 0.0</td>
<td>0.04</td>
<td>0.4 ± 0.40</td>
<td>0.0</td>
</tr>
<tr>
<td>NPBs</td>
<td>0.2</td>
<td>1.6 ± 0.72</td>
<td>5.2</td>
<td>52.4 ± 9.69</td>
<td>1.3</td>
</tr>
<tr>
<td>Tri. cells</td>
<td>1.0</td>
<td>10.10 ± 5.08</td>
<td>0.8</td>
<td>7.7 ± 2.43</td>
<td>3.3</td>
</tr>
<tr>
<td>Quad. cells</td>
<td>0.6</td>
<td>5.7 ± 2.92</td>
<td>0.2</td>
<td>2.4 ± 1.39</td>
<td>1.8</td>
</tr>
<tr>
<td>Apoptic cells</td>
<td>0.4</td>
<td>4.1 ± 1.71</td>
<td>2.1</td>
<td>21.0 ± 3.28</td>
<td>3.9</td>
</tr>
<tr>
<td>Necrotic cells</td>
<td>0.1</td>
<td>1.43 ± 0.73</td>
<td>2.8</td>
<td>27.7 ± 11.07</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Mono. Cell: Mononucleated cell.
Tri. Cell: Trinucleated cell.
Legends as in Table 1.
FIG. 5: Chronic myelogenous leukemia cells in the treatment group exposed to plasma showing a decreased number of immature and progenitor cells, including high incidence of necrotic (Necr) and destructed (D) cells. Table 3 shows that the TR group had a low incidence of binucleated cells and low expression of MNi. In contrast, this group was characterized by high percentages of NPBs with a unique form (multinucleated thread-like bridges) and mononucleated cells (Fig. 6). In the C group, the frequency of the mononucleated cells was 2-fold the binucleated count (Fig. 7). On the other hand, the TR-D2 group showed a nonsignificant difference between the incidences of mononucleated and binucleated cells compared with those in the C group.

Histopathologic examination of bone marrow aspirate demonstrates a shift in the myeloid series to immature forms that increase in number as the patient progresses to the blastic phase of the disease. The marrow is hypercellular, and differential counts of both marrow and blood show a spectrum of mature and immature granulocytes similar to that found in normal marrow. An increased number of eosinophils or basophils are often present, and sometimes monocytosis occurs. The percentage of lymphocytes in both the marrow and blood is reduced compared with normal subjects, and the myeloid ratio in the marrow is usually greatly elevated.

The results recorded in this study confirmed previous investigations, which are represented in Table 1. The TR group showed increases in the ratio of basophils, eosino-
TABLE 4: The incidence of mono-, bi-, tri-, quadrinucleated, apoptic, necrotic cells, and the frequencies of micronuclei and the nucleoplasmic bridges in exposed and non-exposed T- and control groups (counts in 1000 cells).

<table>
<thead>
<tr>
<th>Groups</th>
<th>C- group</th>
<th>T- group</th>
<th>T-D1 group</th>
<th>T-D2 group</th>
<th>T-D3 group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>X ± S.E</td>
<td>%</td>
<td>X ± S.E</td>
<td>%</td>
</tr>
<tr>
<td>Mono. cells</td>
<td>64.2</td>
<td>641.7 ± 18.44</td>
<td>55.2</td>
<td>551.6 ± 112.40</td>
<td>48.9</td>
</tr>
<tr>
<td>Mono. cells + 1 Mn</td>
<td>0.5</td>
<td>4.5 ± 2.36</td>
<td>6.2</td>
<td>61.6 ± 8.16a</td>
<td>7.4</td>
</tr>
<tr>
<td>Mono. cells + 2 Mn</td>
<td>0.0</td>
<td>0.0 ± 0.0</td>
<td>0.1</td>
<td>0.5 ± 0.50</td>
<td>0.0</td>
</tr>
<tr>
<td>BN</td>
<td>32.4</td>
<td>324.1 ± 13.53</td>
<td>17.6</td>
<td>176.0 ± 39.73a</td>
<td>20.9</td>
</tr>
<tr>
<td>BN + 1 Mn</td>
<td>0.7</td>
<td>6.7 ± 0.32</td>
<td>2.2</td>
<td>22.1 ± 7.35</td>
<td>2.7</td>
</tr>
<tr>
<td>BN + 2 Mn</td>
<td>0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0</td>
<td>0.0 ± 0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>NPBs</td>
<td>0.2</td>
<td>1.6 ± 0.72</td>
<td>0.6</td>
<td>6.3 ± 0.37a</td>
<td>5.5</td>
</tr>
<tr>
<td>Tri. cells</td>
<td>1.0</td>
<td>10.10 ± 5.08</td>
<td>0.3</td>
<td>3.0 ± 1.57</td>
<td>1.5</td>
</tr>
<tr>
<td>Quad. cells</td>
<td>0.6</td>
<td>5.7 ± 2.92</td>
<td>0.0</td>
<td>0.0 ± 0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Apoptic cells</td>
<td>0.4</td>
<td>4.1 ± 1.71</td>
<td>2.1</td>
<td>20.6 ± 6.48a</td>
<td>1.9</td>
</tr>
<tr>
<td>Necrotic cells</td>
<td>0.1</td>
<td>1.43 ± 0.73</td>
<td>15.8</td>
<td>158.3 ± 50.02a</td>
<td>10.3</td>
</tr>
</tbody>
</table>

Legends as in Table 2&3
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phils progenitor cells, and myeloblasts, whereas lymphocytes, neutrophils, monocytes, and necrotic cells decreased. On the other hand, the exposed groups showed a decrease in the ratio of mature and immature cells, except the TR-D2 group, which had low eosinophil and basophile percentages without a decrease in lymphocyte and neutrophil counts. However, for all exposed groups there is a tendency toward necrosis, especially for immature cells.

Table 2 discusses the partial improvement in the frequencies of cells in the blood, especially lymphocytes and neutrophils, for the T group, but its results showed high

**TABLE 5:** The levels of Arginase and TGF-β1 in exposed and non-exposed TR- and control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>C group</th>
<th>TR group</th>
<th>TR-D1 group</th>
<th>TR-D2 group</th>
<th>TR-D3 group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
</tr>
<tr>
<td>Arginase</td>
<td>110.27 ± 2.6</td>
<td>439.44 ± 36.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>334.73 ± 7.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>279.82±</td>
<td>507.20 ± 4.40&lt;sup&gt;a,b,c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>1336.7 ± 5500.0</td>
<td>5400.0 ± 57.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>116.82&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Legends as in Table 1

**FIG. 6:** Micrographs of cells from the control group: a normal binucleated cell (A), a binucleated cell with a micronucleus (B), and a binucleated cell with Nucleoplasmic bridge (NPB) (C)
TABLE 6: The levels of Arginase and TGF-β1 exposed and non-exposed T- and control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>C group</th>
<th>T group</th>
<th>TD1 group</th>
<th>TD2 group</th>
<th>TD3 group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
<td>X ± S.E</td>
</tr>
<tr>
<td>Arginase</td>
<td>110.27 ± 2.63</td>
<td>256.58 ± 40.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>231.77 ± 4.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>236.24 ± 2.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>157.25 ± 3.77&lt;sup&gt;a,C,D&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG-β1</td>
<td>1336.7 ± 176.0</td>
<td>5233.3 ± 56.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4833.3 ± 72.72&lt;sup&gt;a,B&lt;/sup&gt;</td>
<td>4800.0 ± 57.80&lt;sup&gt;a,B&lt;/sup&gt;</td>
<td>3340.0 ± 58.87&lt;sup&gt;a,B,C,D&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Legends as in Table 2.

FIG. 7: Micrographs of chronic myelogenous leukemia cells from the treatment group: mononucleated and necrotic cells (A), a mononucleated cell with one micronucleus (B), a binucleated cell (C), a trinucleated cell (D), an apoptotic cell with a micronucleus (E, arrow), necrotic cells (F), a necrotic cell (G, arrow), a necrotic binucleated cell (H, arrow), and a nucleoplasmic bridge (threadlike) with apoptotic daughter cells (I, arrow)

frequencies of immature cells. Exposure to the APPJ minimizes the immature ratio and enhances the necrotic processes.

The CBMN assay is the preferred method for measuring MNi in cultured human
and/or mammalian cells because scoring is specifically restricted to once-divided binucleated cells, which are the cells that can express MNi.\textsuperscript{27} In the CBMN assay, restricting scoring of MNi in binucleated cells prevents confounding effects caused by suboptimal or altered cell division kinetics, which is a major variable in micronuclear assay protocols that do not distinguish between nondividing cells that cannot express MNi and dividing cells that can.\textsuperscript{28} Therefore, in this study, we investigated CML cases using the CBMN assay, which is considered a comprehensive system for measuring DNA damage, cytostasis, and cytotoxicity. This assay included scoring of mononucleated, binucleated, trinucleated, quadrinucleated, necrotic, and apoptotic cells as well as MNi and NPBs (Tables 3 and 4). The TR and T groups scored unexpected low MNi frequencies, but this is interpreted as a result of the high mitotic division rates of the CML cells and their intolerance to the arresting action of cytochalasin B. Accordingly, the expression

\textbf{FIG. 8:} Micrographs of chronic myelogenous leukemia cells from the treatment group: a mononucleated cell (A), a binucleated cell with a micronucleus (B), a binucleated cell with a necrotic daughter cell and a mononucleated cell with a micronucleus (C, arrow), a deformed binucleated cell (D, arrow), a NPB with necrotic daughter cells (E, arrow), a nucleoplasmic bridge (NPB) (F, arrow), and multinucleated NPBs (G and H)
FIG. 9: Micrographs of chronic myelogenous leukemia cells from the treatment group after plasma jet exposure: apoptotic cells (A, arrows), a nucleoplastic bridge (NPB) (B, arrow), necrotic binucleated cells (C and D, arrows), a necrotic cell (E, arrow), a destructive NPB (F, arrows), apoptotic cells (G, arrow), a multinucleated cell with a destructive NPB (H, arrow), a destructive NPB without daughter cells (I, arrow), a destructive NPB with a necrotic cell (J, arrows), an apoptotic binucleated cell (K, arrow), and an apoptotic and necrotic cell (L, arrows). There is a significant reduction in NPB counts in the treatment-resistant (TR) group when compared with their percentages in the TR-D1 and TR-D3 groups. Trinucleated and quadrinucleated cells scored high frequencies in TR groups when compared with those in the in TR and control groups.
of the MNi were very low. Expression of MNi could be a marker of DNA hypomethylation. For example, the DNA methylation inhibitor 5-azacytidine induces condensation of heterochromatin regions of chromosomes 1, 9, and 16 and the specific loss of these chromosomes as MNi in human lymphocytes.

Data in Table 3 show that the TR group did not respond to cytochalasin B and its arresting action (mitotic division was uncontrolled), with low incidence of MNi expression; APPJ exposure changed the ratio of mononucleated to binucleated cells, especially for the TR-D2 group. This reflects that the CML cells were controlled after the exposure, their division rate decreased, and MNi expression increased within the binucleated cells. The same trend was scored for the T group and its exposed groups (Table 4), especially the T-D2 group.

The data in Table 3 point to the presence of a characteristic shape of NPBs (multinucleated and thread-like anaphase bridges), and their counts were significantly higher in the TR group than in the C, TR-D1, and TR-D3 groups. The NPB count in the TR-D2 group was not significantly different than that of the TR group, but the NPBs did differ in shape (fragmented threads, as shown in Fig. 8). As shown in Table 4, the traditional forms of NPBs and the anaphase bridges were observed in the T group, and their counts gradually decreased in the T-D1 to T-D3 groups.

Fenech proposed that NPBs between nuclei in binucleated cells should be scored using the CBMN assay because they provide a measure of chromosome rearrangement that is otherwise not achievable in this assay if only MNi are scored. NPBs occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell during anaphase. Rarely is it possible to observe dicentric anaphase bridges before the nuclear membrane is formed because cells proceed rapidly through anaphase and telophase, completing cytokinesis, which ultimately results in breakage of the NPB when the daughter cells separate. However, in the CBMN assay, binucleated cells with NPBs are allowed to accumulate because cytokinesis is inhibited, and the nuclear membrane is eventually formed around the chromosomes, allowing an anaphase bridge to be observed as an NPB.

Various mechanisms could lead to NPB formation following misrepair of strand breaks in DNA. Typically, a dicentric chromosome and an acentric chromosome fragment are formed, resulting in the formation of an NPB and a mononucleus, respectively. Scoring NPBs is important because it provides direct evidence of genome damage resulting from misrepaired DNA breaks or telomere end fusions, which are otherwise not possible to deduce by scoring only MNi, which could originate from either acentric chromosome fragments or chromosome loss.

In Table 3 the data show that there is a gradual increase in the sum of the apoptotic and necrotic cells in the APPJ-exposed groups (TR-D2 and TR-D3), but there are significant increases in apoptotic cell counts in the T-D2 and T-D3 groups compared with the T and T-D1 groups (Table 4). The necrotic cell percentages were insignificantly decreased in the T-D1, T-D2, and T-D3 groups compared with the count in the T group.

The proliferative status of the viable cells and their cytostatic arrest may be represented within the discussion of the increase in trinucleated and quadrinucleated cell

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percentages in the APPJ-exposed groups (Table 3) and in the T-D1 and T-D2 groups (Table 4). These cell counts were very low in the TR and T groups in comparison with their ratio after APPJ exposure.

The major cause of antitumor chemotherapy failure is the tumor developing multidrug resistance. Therefore, the effect of HV pulsed, cold atmospheric plasma jets, which are chemically activated with oxygen, on B16 tumor cells and COLO320DM multidrug-resistant cells (human colon cancer cell line). The results show an increased rate of apoptosis compared to chemotherapy treatment alone. The effects of cold plasma are caused by charged particles, reactive oxygen species, reactive nitrogen species, ultraviolet photons, and the intense electric field. To obtain more efficient action on mammalian cells (useful for cancer therapy), they used chemically activated cold plasma (a helium and oxygen gas mixture). The effect of chemically activated cold plasma (apoptosis or necrosis) depends on the gas mixture and treatment duration.32

Apoptosis is important not only in the turnover of cells for generation in all types of tissues but also during the normal development, differentiation, and senescence of

FIG. 10: Micrographs of chronic myelogenous leukemia cells from the treatment-resistant group after plasma jet exposure: necrotic cells with destructive nucleoplasmic bridges (NPBs) (A, arrows), an apoptotic cell (B, arrow), a necrotic cell (C, arrow), a destructive NPB (D, arrow), multinucleated cells with destructive NPBs (E–G, arrow in E), an apoptotic cell and destructive NPB (H, arrows), a binucleated cell with three micronuclei (I, arrow), a binucleated cell with a necrotic daughter cell and an apoptotic cell (J, arrow), a necrotic cell and a destructive NPB (K, arrows), a binucleated cell with one micronucleus (L, arrow), a binucleated cell and necrotic cell (M, arrows), and a quadrinucleated cell (N, arrow). BN, binucleated cell; MN, micronucleus; Mono, mononucleated cell; NPB, nucleoplasmic bridge; SE, standard error; Tri, trinucleated cell; Quad, quadrinucleated cell.
organisms. The plasma jet has been proposed as a novel therapeutic method for anticancer treatment. Treatment with air or nitrogen plasma jets caused apoptotic death in human cervical cancer He La cells. This has been described as being caused by the generation of reactive oxygen species, which function as surrogate apoptotic signals by targeting the mitochondrial membrane potential. Antioxidants or caspase inhibitors ameliorated the apoptotic cell death induced by air and nitrogen plasma jets, suggesting that the plasma jet may generate reactive oxygen species as a proapoptotic cue, thus initiating mitochondria-mediated apoptosis.

The main characteristic of a plasma source for use in medical therapy is its direct application on or in the human body. Therefore plasma effects on mammalian cells are of basic interest, and in nitro tests to characterize plasma–cell interactions should include characterization of basic cellular parameters such as morphology, viability, or proliferative characterization of special cellular responses, including the influence on DNA or cellular protein.

Table 5 shows that the arginase and TGF-β1 concentrations were 3.9– and 4.1-fold more, respectively, in the TR group than in the C-group. On the other hand, except for the TR-D3 group, there were decreases in the concentrations of arginase and TGF-β1 in the APPJ-exposed groups (the TR-D1 and TR-D2 groups). As shown in Table 6, however, the arginase concentration was higher in the T group than in the C group by 2.3-fold. The TGF-β1 concentration in the T group was 3.9-fold higher than in the C group. The APPJ-exposed groups had decreased concentrations of arginase and TGF-β1, although there was an insignificant increase in arginase only at D2 compared with D1.

The importance of arginase may be in the production of ornithine for the synthesis of the polyamines putrescine and spermine, which are required for normal cellular proliferation. Several reports indicate that higher arginase activity is present in cancerous tissues, which is different than in normal tissues. Except in liver tissue, arginase is also present in many human tissues and in circulating blood cells, especially erythrocytes and macrophages. The cells use L-arginine to synthesize nitric oxide (NO) and polyamines through the inducible NO synthase and arginase. The released NO contributes to the tumoricidal activity of macrophages, whereas polyamines promote the growth of tumor cells.

The TGF-β superfamily encompasses widespread and evolutionarily conserved polypeptide growth factors that regulate and orchestrate the growth and differentiation of all cell types and tissues. While they regulate asymmetric cell division and determine cell fate during early development and embryogenesis, TGF-β family members play a major regulatory role in hormonal and immune responses, cell growth, cell death, cell immortalization, bone formation, tissue remodeling and repair, and erythropoiesis throughout adult life. The biological and physiological function of TGF-β, the founding member of this family, which includes TGF-β1, and its receptors are of central importance to human disease, particularly cancer. By regulating cell growth, death, and immortalization, TGF-β signaling pathways exert tumor suppressor effects in normal cell and early carcinomas. Thus it is not surprising that a large number of human tumors arise due to mutations or deletions in the gene coding for the various TGF-β signaling
components. As tumors develop and progress, these protective and cytostatic effects of TGF-β are often lost. TGF-β signaling then switches to promote cancer progression, invasion, and tumor metastasis.

There is high expression of genes related to drug resistance and inhibition of cell apoptosis. To address the mechanism of CD133-positive tumor cells showing strong resistance to therapeutic drugs, both CD133-positive cells and CD133-negative cells were collected to investigate the expression of multidrug resistance– and DNA mismatch repair–related genes, as well as genes related to the inhibition of cell apoptosis, within these 2 populations. BCRP1 has been demonstrated to play an important role in the resistance of normal stem cells and tumor stem cells to drugs.37,38 In addition, the presence of the DNA repair protein MGMT has been demonstrated to render cells resistant to cytotoxic actions of methylating and chloroethylating agents, such as tomozolomide.39,40

Antiapoptotic genes, including FLIP, BCL-2, and BCL-XL, also were found at significantly higher levels in CD133-positive cells than in antilogous CD133-negative cells. The inhibitor of apoptosis protein family blocks cell death; IAP family members XIAP, CIAP1, CIAP2, NAIP, and survival have higher expression levels on CD133-positive cells than CD133-negative cells.41

The principal cause of treatment failures in CML is the emergence of multidrug resistance. The mechanisms of resistance include defects in apoptotic and necrotic pathways. All former genes represent some of the genes that play a critical role in incidence and treatment of CML such as P53 and Bcl-2.

There is a need to develop new approaches that enhance the elimination of malignant cells. One promising method for treatment of CML, and cancers in general, is the effect of nonthermal plasma; this is apparent from the results of this study. The effect of nonthermal plasma on cancer cells is caused by all products resulting from the plasmas, including free radicals, ultrasonic and electromagnetic waves, and electromagnetic fields, which enact their effects by upregulating and downregulating associated genes. These effects finally increase the apoptosis and necrosis of malignant cells. An important rule for any antitumor agent is blocking the prometastatic arm of the TGF-β signaling pathway without affecting its tumor-suppressive effects.42

Finally, based on data presented in this study, an APPJ can be used for biochemical and cytogenetic remission of patients with CML, especially those resistant to or intolerant of chemotherapy. The duration of 80 seconds was the most effective and safe dose of APPJ treatment.43 Moreover, the presence of a low incidence of binucleated cells for CML in CBMN assay does not mean a low nuclear division index; it does, however, mean that control and governance of cytochalasin B in mitotic division is very low (high division rate). Therefore, for CML cells, a high ratio of binucleated cells is expressed and observed only after exposure to the APPJ.

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and Internal Medicine, Kasr Al Ainy Hospital, Cairo University, for acting as a medical consultant, providing facilities, and performing blood sampling for this study.

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