Response of S180 murine tumor to bleomycin in combination with radiation and hyperthermia using micronucleus assay: A multimodality approach for therapeutic augmentation

B S Satish Rao & P Uma Devi*
Department of Radiobiology, Kasturba Medical College Manipal, Manipal 576 104, India
*Department of Research, Jawaharlal Nehru Cancer Hospital & Research Centre, Idgah Hills, Bhopal 462 001, India

Received 15 June 2004; revised 24 March 2005

Response of a transplantable tumor, S180, grown intradermally in inbred Balb/c mice, was assessed by using micronucleus assay after treating the solid tumors with bleomycin (BLM), radiation (RT) and hyperthermia (HT) vis-a-vis multimodality approach. The frequency of micronuclei (MN) though did not vary greatly during the one week of observation in untreated tumors, it significantly increased in the drug and RT groups at 24 hr post-treatment. However, MN frequency was non-significant in the HT group from the control. A drug dose dependent linear increase in the frequency of MN induction was evident in 10, 15 and 20 mg/kg body weight BLM alone treated groups. Combination of radiation with BLM or HT further increased the MN counts in the bimodality groups. But, MN induction at 24 hr post-treatment in the trimodality group (BLM+RT+HT) was non-significant from that of the bimodality treatments. However, the tumors treated with trimodality treatment presented severe tumor necrosis, indicating increased cell loss, and resulting in immediate tumor regression. In all the bi-modality groups MN counts though declined 3 or 5 days post-treatment, the values remained significantly higher than the control, on day 7 post-treatment. Micronucleus assay could be used as a predictive parameter for the assessment of post-irradiation tumor regression response. However, the tumor response assessment with MN assay alone may not be sufficient and the role of other parameters, such as apoptosis and necrosis, in immediate tumor regression, especially radiosensitive/thermosensitive tumors can not be ignored while taking multimodality approach into consideration for cancer therapy.

Keywords: Bleomycin, Hyperthermia, Micronuclei, Multimodality treatment, Radiation

In cancer therapy, patient intolerance and the drug toxicity are the major limiting factors for the therapeutic armamentarium such as radiotherapy and chemotherapy. Therefore, multimodality approach has been appreciated using the combination of radiation, chemotherapeutic drugs and hyperthermia at tolerable doses to combat the disease. Earlier pre-clinical studies using murine transplantable tumors and clinical studies have demonstrated the efficacy of such a multimodality approach using various chemotherapeutic drugs in combination with either radiation and/or hyperthermia

Bleomycin (BLM), an antibiotic is reported to have antitumor activity and is used in clinics extensively against glioma and nasopharyngeal carcinoma. Hyperthermic sensitization of BLM induced cell killing resulting in enhanced cell killing and tumor regression and BLM's potential to enhance the effect of radiation have been reported. The mechanism of action of BLM has been attributed to its ability to induce DNA strand breaks. It is understood that non-repair or inhibition of the DNA double strand break repair contributes to chromosomal aberrations, which could be analysed by the quantitative analysis of micronuclei. These micronucleated cells may complete few divisions before they are finally eliminated. Therefore, micronucleus assay could be a very useful parameter for the prediction and prognosis of the tumor treatment. The present study is an attempt to understand the response of a transplantable murine tumor, S-180, to BLM, radiation and hyperthermia as single, bi- and trimodality treatments, using micronucleus assay as an experimental endpoint.

Materials and Methods
The murine tumor, Sarcoma 180 (S180), originally obtained from the Advanced Center for Treatment,
Research and Education in Cancer (ACTREC), Tata Memorial Center (TMC), Navi Mumbai, was propagated in inbred Balb/c mice by serial transplantation in ascites form, injecting 10^6 viable cells into the intraperitoneal cavity of female mice. Solid tumors were produced by intradermal injection of viable cells (5 x 10^5) on the dorsum of the host animals, as described earlier. Once the tumors attained 100 ± 10 mm^3, they were used for the experiments.

Animals were anesthetized (Ketamine, 50 mg/kg and Diazepam, 0.5 ml/animal) and tumors were irradiated locally using ^60^Co teletherapy unit (Siemens, Germany, routinely used for patient treatment at The Department of Radiotherapy and Oncology), at a dose rate of 1 Gy/min (RT). The dosimetric calculations were done by Prof. J.G.R. Solomon, Radiation Physicist, Department of Radiotherapy and Oncology, Kasturba Medical College, Manipal. For hyperthermia treatment (HT), tumors were heated locally using a thermostat controlled water bath (Julabo, Germany) at 43°C for 30 min as explained earlier.

**Treatment schedule and sequence** — The drug was injected 15 min before RT or 30 min before HT; RT was immediately followed by HT. For the trimodality treatment, BLM was injected (ip) 15 min before local RT, followed immediately by HT.

**Micronucleus (MN) assay** — At different time points after treatments, animals were anesthetized with ether and tumors were excised into Dulbecco’s Minimal Essential Medium (DMEM). Single cell suspension was prepared mechanically as described earlier and fixed in carnoy’s fixative. MN slides were prepared by dropping the tumor cell suspension on to grease free slides. Slides were coded, stained with ethidium bromide and MN frequency was scored in 2000 cells per animal using fluorescence microscope under UV excitation as described earlier. Each group contained 5 animals.

**Statistical analysis** — The drug dose response curve for MN frequency at doses 10, 15 and 20 mg/kg body weight was fitted on a linear model (Y = α+βX) and the MN frequency was compared with the control and among the different groups by Student’s t test using GraphPAD InStat, Software, USA.

**Results**

The frequency of spontaneously occurring MN did not vary greatly during the one week of observation (Table 1). The frequency of MN in the tumors left untreated was in the range of 3.2 to 3.5% on day 1 to day 7. The MN counts at 24 hr after treatment was significantly (P<0.05) higher in all the drug and RT groups, while HT treatment did not produce any significant change from control value. BLM (10, 15 and 20 mg/kg body weight) alone, at 24 hr post-treatment showed a drug dose-dependent linear (r² = 0.9170) increase in the frequency of MN induction. Increase in the BLM dose from 10 mg/kg to 15 mg/kg and 15 to 20 mg/kg significantly (P<0.01) increased the MN counts. Bleomycin, 10 mg/kg body weight was selected for further combination treatments with radiation or hyperthermia.

In all the single modality treatment groups, the MN count increased with time after treatment and reached a maximum on 3 or 5 days post-treatment and the values remained significantly (P<0.01) higher than control even on day 7 post-treatment. On day 5, when the highest MN count was recorded, BLM (10 mg) produced the maximum MN, although this was not significantly different from that produced by 10 Gy radiation, but was significantly (P<0.05) higher than that induced by 43°C, 30 min.

The combination of BLM with RT at 24 hr post-treatment produced significantly (P<0.0001) higher MN counts when compared to BLM alone, while with 10 Gy gamma radiation the increase was not significant. All the other bimodality treatments, produced significantly (P<0.05) higher MN counts at 24 hr post-treatment compared to the individual agents used in the combination. The MN induction after RT+HT treatment was significantly higher than the combination of BLM with RT (P<0.05) or 43°C, 30 min (P<0.005). The combination of all the three modalities did not result in any increase in MN frequency over that of the bimodality treatments. However, in this group there was severe necrosis of the tumors immediately after the treatment, indicating increased cell loss.

Bleomycin or 10 Gy combination with 43°C, 30 min produced maximum MN counts on day 3. The frequency of MN could not be followed after 3 days in the animals treated with BLM + 43°C, 30 min due to severe necrosis in those tumors. In all the treatment groups the MN count declined after day 3 or day 5 post-treatment, though the values remained significantly (P<0.01) higher than in control on day 7 post treatment. The maximum MN after 10 BLM +10 Gy treatment was reached on day 5, which was
These micronucleated cells may be metabolically altered, with a primary action of HT in the mitotic cell, leading to some time and MN may be produced in more than one post treatment mitosis. Such a phenomenon can explain the high MN counts at 3-7 days after exposure after various treatments in the present study.

The cytotoxicity of BLM is generally correlated with its ability to induce DNA strand breaks. Strand scission is the first and most commonly observed result of BLM action on DNA. Miyaki et al. have demonstrated that at lower drug concentrations single strand breaks occur, while higher concentrations produce double strand breaks. A linear increase in the frequency of MN after treatment with different doses of BLM (10 to 20 mg) observed in the present study could arise from an increased number of chromosome aberrations resulting from DNA double strand breaks.

The present observation that moderate heat treatment did not bring about any immediate detectable increase in MN count, but a significant increase occurred at 3-7 days with the maximum count during day 3 post-treatment, is similar to the findings of Falkvoll and Real on human melanoma xenografts. Though a similar trend in the MN induction was seen in the present study, the intensity of damage was less severe. This may be due to the different types of tumors. Streffer suggested that the primary action of HT in the mitotic cell may be on proteins affecting the spindle apparatus. This can interfere with the normal cell division, leading to

---

Table 1—Frequency of micronuclei, 1, 3, 5 and 7 days after treatments with BLM, radiation and hyperthermia in relation to various combination treatments

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>3.20 ± 0.09</td>
<td>3.28 ± 0.08</td>
<td>3.34 ± 0.10</td>
<td>3.50 ± 0.03</td>
</tr>
<tr>
<td><strong>Single modality treatments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLM alone (mg/kg, b.wt)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.10</td>
<td>3.60 ± 0.03*</td>
<td>4.60 ± 0.23</td>
<td>6.66 ± 0.40</td>
<td>4.30 ± 0.11</td>
</tr>
<tr>
<td>3.15</td>
<td>4.52 ± 0.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.20</td>
<td>5.18 ± 0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperthermia alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.43°C, 30 min</td>
<td>3.28 ± 0.06*</td>
<td>5.38 ± 0.07</td>
<td>5.36 ± 0.10</td>
<td>4.52 ± 0.22</td>
</tr>
<tr>
<td>Radiation alone</td>
<td>4.76 ± 0.23*</td>
<td>5.60 ± 0.12</td>
<td>5.92 ± 0.07</td>
<td>4.26 ± 0.19</td>
</tr>
<tr>
<td><strong>Bimodality treatments</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.10 mg/kg BLM + 43°C, 30 min</td>
<td>4.52 ± 0.17*</td>
<td>5.88 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.10 Gy + 43°C, 30 min</td>
<td>5.38 ± 0.08*</td>
<td>5.72 ± 0.06</td>
<td>4.82 ± 0.11</td>
<td>4.70 ± 0.24</td>
</tr>
<tr>
<td>9.10 mg/kg BLM + 10 Gy</td>
<td>5.02 ± 0.10*</td>
<td>5.92 ± 0.51</td>
<td>6.84 ± 0.09</td>
<td>6.22 ± 0.14</td>
</tr>
<tr>
<td><strong>Trimodality treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.10 mg/kg BLM + 100 Gy + 43°C, 30 min</td>
<td>4.92 ± 0.22*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All the treatment groups significant (P<0.05) when compared to the respective control groups, except on day 1, group 1 with 5 (NS), 9 with 6 (NS), group 4 significant with group 2 & 3 (P<0.05); group 3 significant with group 2 (P<0.01); On day 3: group 8 with 6(NS); On day 5: group 9 with 2 (NS). * Data taken from Uma Devi and Rao, reproduced with permission.

Interestingly, (P<0.01) higher than in 10 Gy + HT group. However, it was not significantly higher than that produced by 10 BLM alone. The MN counts remained almost the same on day 7 post-treatment in all the treated groups, except in BLM+RT, which was significantly (P<0.005) higher than in the other bimodality treatments.
cytogenetic abnormalities. In the present study, tumors treated with HT alone or its combination with other agents resulted in immediate shrinkage/blackening of the tumors. This may be due to the severe necrosis or apoptotic cell death. At the later time points the expression of the cytogenetic damage reached maximum on day 3 or 5 (indicated by increased MN counts) followed by decreased MN counts. The decrease in the MN counts may be due to the elimination of the micronucleated cells and also by the dilution caused by the addition of the new cells to the existing tumor mass.

The chemosensitizing effect of hyperthermia is more pronounced than its radiosensitizing effect on the S180 tumors. The increase in the MN count at 24 h post treatment in the bimodality groups compared to that produced by BLM, RT or HT alone indicates an interaction of the two agents at the level of DNA. HT treatment is known to interfere with the repair of RT induced primary damage to DNA\(^ {27-29}\), resulting in increased cytogenetic damage. Similarly, the inhibition of repair of the BLM induced DNA damage by heat treatment could lead to fixation of the damage and enhanced cell killing, resulting in potentiation of the drug effect. Terasima et al.\(^ {30}\) have demonstrated the ability of mammalian cells to repair BLM induced DNA damage. Single strand breaks are repaired within 3 hr, while double strand breaks take longer time to repair\(^ {31}\). In the present study application of heat within the first hour may have inhibited the repair of both single strand breaks and double strand breaks, which resulted in enhanced chromosomal damage, expressed in the form of increased MN frequency.

The trimodality treatment in the present study, did not show any significant increase in the MN count compared to some of the bimodality treatments. This may be due to the severe damage to the cells resulting in immediate cell death. It is possible that at 24 hr post treatment, only a small fraction of the cells entered division and contributed to the MN. Even these cells may not have been able to continue proliferation as indicated by the progressive shrinkage and complete regression of the tumor in the trimodality treatment groups.

In recent years, the utility of micronuclei as a predictive parameter for the prognosis of tumor during and post-treatments situations has gained considerable interest in clinics. The micronucleus assay is useful for prediction and prognosis of resistant tumors and while assessing the single agent treatment response. However, this assay by itself may not be sufficient while handling sensitive tumors or in a multimodality approach. Therefore, the additional parameters such as apoptosis and necrosis are needed to be included while assessing the treatment response of sensitive tumors as well as after multimodality treatments.

**Acknowledgement**

The financial assistance to BSSR from ICMR, New Delhi is acknowledged. Thanks are due to Prof. Koteshwar Rao and Prof. J. G. R Solomon, Department of Radiotherapy and Oncology, Kasturba Medical College, Manipal, for necessary irradiation facilities and dosimetric calculations, respectively.

**References**


